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The PP1 Regulatory Subunit, Inhibitor-2 Induces a Mitotic Cell Cycle Arrest in *Xenopus* Cycling Egg Extracts that is Dependent on the MAPK Pathway

By

Arian Khandani

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Masters of Science

Department of Cell Biology

Edmonton, Alberta

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Ellen K. Shibuya

Hanne L. Ostergaard

Manijeh Pasdar

August 29, 2003

Date

Abstract

I have examined the role of protein phosphatase-1 (PP1) in the regulation of the *Xenopus* cell cycle by using a specific inhibitor of PP1, Inhibitor-2 (Inh-2) protein. I showed that the addition of rabbit Inh-2 protein to a *Xenopus* cycling egg extract induces M-phase arrest of the cell cycle. Interestingly, this M-phase arrest was dependent on p42MAPK activation. Since p90Rsk phosphorylated Inh-2, Inh-2 was thiophosphorylated to determine the role of this phosphorylation in M-phase arrest. I found that p90Rsk-thiophosphorylated Inh-2 was not more effective than unphosphorylated Inh-2 protein in inducing M-phase arrest. Human Inh-2 protein that lacks potential p90Rsk phosphorylation consensus sites also induced an M-phase arrest. Thus, although these results suggest that one way for PP1 inactivation to induce an M-phase arrest is by activation of the p42MAPK pathway (including pp90Rsk), phosphorylation of Inh-2 by p90Rsk is not solely required to sustain the induced M-phase arrest.

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At the end I would like to dedicate this thesis, to my grandma who passed away due to Parkinson disease during my study here.

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List of Abbreviations:

Ala	Alanine (A)
Arg	Arginine (R)
Asn	Asparagine (N)
Asp	Aspartic acid (D)
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAF-1	Chromatin assembly factor-1
CKII	Casein kinase II
CaMKII	Ca^{++}/Calmodulin-dependent protein kinase II
CC	Chromosome condensation
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CSF	Cytostatic Factor
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular signal Regulated Kinase
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*,*N*'-tetraacetic acid
G1	Gap 1 phase
G2	Gap 2 phase
GSK3	Glycogen synthase kinase-3
GVBD	Germinal vesicle breakdown
HCG	Human chorionic gonadotropin
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
Hr	Hour(s)
IgG	Immunoglobulin G
Inh-2	Inhibitor-2
IP	Immunoprecipitation
IU	International unit
kDa	Kilodalton

LB	Luria-Bertani broth
Lys	Lysine (K)
M-phase	Mitotic phase
MAPK	Mitogen Activated Protein Kinase
p42MAPK	p42 Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MEK(QP)	Constitutively-active MEK protein
MII	Metaphase II
Min	Minute(s)
MPF	Maturation Promoting Factor
MW	Molecular weight
NEBD	Nuclear Envelope Breakdown
p90Rsk	p90 Ribosomal S6 kinase
pp90Rsk	Phosphorylated p90Rsk
PP1	Protein phosphatase-1
RPM	Revolutions per minute
S-phase	DNA synthesis phase
SAC	Spindle Assembly Checkpoint
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec	Second(s)
Ser	Serine (S)
Thr	Threonine (T)
v/v	Volume per volume

CHAPTER 1:

Chapter 1: Introduction

1.1 Overview of Cell Cycle

The study of the cell cycle involves understanding the molecular mechanisms that regulate the progression through different stages of cell division. In general, the cell cycle is divided into four phases, gap 1 (G1), DNA synthesis (S-phase), gap 2 (G2) (where G1, S-phase, and G2 comprise interphase), and mitosis (M-phase). In G1 and G2 phases, the cell monitors conditions for continuance of the cell cycle. S-phase is when the DNA replication takes place in the nuclei. M-phase is subdivided into prophase, prometaphase, metaphase, anaphase, and telophase. At the end of M-phase, the actual cell division takes place and one copy of each chromosome segregates into each daughter cell. In general, interphase occupies most of the length of one cell cycle and M-phase is usually the shortest phase (Murray and Hunt, 1993).

1.1.1 *Xenopus* Model System

The amphibian system, specifically the *Xenopus laevis* model system, is a well-established system used to study cell cycle events. *Xenopus laevis* is an African clawed frog, and its eggs and oocytes (immature eggs) are used to study the cell cycle. Some of the advantages for using this system are that large quantity of *Xenopus* eggs or oocytes can be obtained from *Xenopus* that are naturally arrested at a specific phase of the cell cycle, and then natural or artificial means can be used to release the oocytes/eggs from arrest. These oocytes/eggs can provide large quantities of extract for use in biochemical experiments. *Xenopus* oocytes/eggs are also very large (approximately 1 mm in diameter), resistant to injury, and suitable for microinjection experiments (Murray, 1991). Another important advantage of this system is the establishment of cell-free systems using *Xenopus* oocyte and egg extracts that reproduce cell cycle events *in vitro,* as they would occur naturally *in vivo* (Lohka and Masui, 1983; Lohka and Maller, 1985; Murray and Kirschner, 1989; Shibuya *et al.*, 1992).

Figure 1 shows a schematic diagram of *Xenopus* oocyte maturation. Immature oocytes are arrested in G2 of meiosis. Meiosis is a specialized cell cycle that will

produce gametes with a haploid amount of DNA. Hormonal stimulation (progesterone in the case of *Xenopus*) causes immature oocytes to undergo meiotic maturation, the process by which an immature oocyte develops into a mature, fertilizable egg. After progesterone stimulation, G2-arrested oocytes enter into M-phase. This entry is monitored by nuclear envelope breakdown which is the first visible change in oocytes and is referred to germinal vesicle breakdown (GVBD). On completion of maturation, the egg is arrested at metaphase of the second meiotic division. The egg remains arrested at this stage until fertilization. Fertilization releases the egg from M-phase arrest and allows the zygote (fertilized egg) to enter interphase and start the mitotic, embryonic cell cycles (Murray and Hunt, 1993). The release of the eggs from M-phase arrest is dependent on the increase of intracellular Ca^{++} ions and requires activation of Ca^{++}/Calmodulin-dependent protein kinase II (CaMKII) activity (Lorca *et al.*, 1993). The cell cycle of early *Xenopus* embryos is simple, rapid and consists of only S and M-phases to accommodate the segmentation of the embryo.

Xenopus oocyte maturation exhibits many similarities to mammalian oocyte maturation. For instance, in both species oocytes are arrested in G2 phase, require hormonal stimulation to be released from the arrest, and they both undergo similar physiological changes upon entry into M-phase. In addition, on completion of maturation, *Xenopus* and mammalian mature eggs are arrested at metaphase of the second meiotic division, the stage at which fertilization normally occurs (reviewed by Nigg, 1993; Abrieu *et al.*, 2001).

Thus, the *Xenopus laevis* embryonic system has been the choice of many laboratories for studying cell cycle events because of its many similarities with the mammalian embryonic cell cycle and importantly, its experimental advantages.

1.1.2 Discovery of Maturation Promoting Factor (Cyclin B/Cdc2)

Two families of proteins, Cyclin dependent kinases (Cdks) or Cell division cycle (Cdc) and Cyclin proteins are the major regulators of cell cycle progression. Association of Cyclins with Cdk (Cdc) proteins regulates the progression of the cell cycle. The first Cyclin/Cdk complex to be identified was the mitotic Cyclin B/Cdc2 complex. This complex was first discovered as an activity called Maturation

Promoting Factor (MPF) (Masui and Markert, 1971). When the cytoplasm of a maturing frog oocyte was microinjected into an immature oocyte, the recipient oocyte underwent meiotic maturation without any hormonal stimulation and arrested in metaphase of meiosis. Thus, because the complete process of maturation was induced by this cytoplasmic activity, it was named Maturation Promoting Factor (MPF).

MPF was later found to be a regulator of entry into M-phase both during meiosis and mitosis (Wasserman and Smith, 1978). The cytoplasm of frog embryos at different times after fertilization was assayed (every 15 to 30 min up to 5 hr) for MPF activity by microinjection into an immature oocyte. MPF activity appeared in the cytoplasm of embryos shortly before division, and this activity oscillated during the mitotic cell cycles of amphibian embryos. Since these initial studies, it has been found that MPF is a universal regulator required for the transition from interphase into M-phase of the cell cycle in all eukaryotic cells studied to date (reviewed by Nebreda and Ferby, 2000).

In 1988, Lohka and colleagues biochemically purified MPF from *Xenopus* egg extracts. The purified MPF consisted of two proteins, 32 kDa and 45 kDa, and fractions containing purified MPF were able to phosphorylate the exogenous substrate histone H1 *in vitro*. The 32 kDa protein was identified as Cdc2 kinase (also referred to as Cdk1) by immunoblotting and immunoprecipitation of purified MPF with anti-yeast Cdc2 antibody (Gautier *et al.*, 1988). Using the same approach with anti-*Xenopus* Cyclin B antibody, the 45 kDa protein in purified MPF was identified as Cyclin B protein (Gautier *et al.*, 1990).

In summary, MPF is a universal factor that drives the cell cycle from interphase into M-phase both during meiosis and mitosis in all eukaryotic cells. It consists of a complex of Cyclin B protein and Cdc2 kinase. MPF activity oscillates during the cell cycle with high activity during M-phase and low activity during interphase.

1.1.3 Regulation of Cyclin Dependent Kinases (Cdk) and Cyclins

We now know that association of different members/member of these two families of proteins regulates passage through each phase of the cell cycle in all

eukaryotic cells. Cyclins are the regulatory subunits and do not have any enzymatic activity. Cyclins, as the name implies, are members of a family of proteins that are continuously synthesized but periodically degraded (reviewed by Hunt, 1989; Irniger, 2002). In general, G1 Cyclins include Cyclin E and D, which are synthesized during G1 phase and are required for entry into S-phase. Although, Cyclin A and B were first categorized as mitotic Cyclins that were required for entry into M-phase, Cyclin A was also found to be required during S-phase (reviewed by Pines, 1993). Cdks (Cyclin dependent kinases), a subfamily of the Cdc (Cell division cycle) proteins, are the catalytic subunits which function as serine/threonine (Ser/Thr) kinases. The first Cdk (Cdk1/Cdc28/Cdc2) was originally identified in the yeast and it was found later that higher eukaryotes contain additional Cdk proteins. Cdk2, 4 and 5 were found to be required during G1 and S-phases (reviewed by Nigg, 1993; Pines, 1993). In the *Xenopus* embryonic system, Cyclins A, B, and E as well as Cdk1 and 2 have already been described (reviewed by Ferrell, 1999b).

Figure 2 shows a schematic diagram of the regulation of Cyclin B/Cdc2 kinase during the cell cycle in the *Xenopus* embryonic system. As Cyclin B protein is synthesized during interphase, it associates with Cdc2 kinase. The complex is kept inactive during interphase by Myt1 and Wee1 kinases that phosphorylate threonine 14 and tyrosine 15, respectively of Cdc2 kinase (Mueller *et al.*, 1995a; Mueller *et al.*, 1995b). *Xenopus* Wee1 phosphorylates only the tyrosine 15 residue of Cdc2, since mutated Cdc2 (Y15F) cannot be phosphorylated by this kinase (Mueller *et al.*, 1995a). In addition, recombinant *Xenopus* Myt1 can phosphorylate both threonine 14 and tyrosine 15 residues of Cdc2 kinase *in vitro* (Mueller *et al.*, 1995b). Cdc2 kinase is phosphorylated by Cdc2 Activating Kinase (CAK) on threonine 161, and phosphorylation of this site is required for full activation of Cdc2 kinase (reviewed by Harper and Elledge, 1998).

The activation of Cyclin B/Cdc2 kinase is accomplished by Cdc25, a dual specificity protein phosphatase that dephosphorylates the inhibitory phosphorylated sites of Cdc2 (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991; Honda *et al.*, 1993). Cdc25 was able to dephosphorylate the tyrosine residue of Cdc2 that had been phosphorylated *in vitro* (Gautier *et al.*, 1991). Addition of Cdc25

protein to *Xenopus* oocyte extracts also dephosphorylated Cdc2 kinase as analyzed by anti-phosphotyrosine antibodies (Kumagai and Dunphy, 1991). Further, addition of human Cdc25 protein to the purified, inactive Cyclin B/Cdc2 complex caused activation of this complex *in vitro* (Strausfeld *et al.*, 1991). The activation was measured by phosphorylation of histone H1 protein by Cyclin B/Cdc2 *in vitro* (Strausfeld *et al.*, 1991). Phosphoamino acid analysis was used to identify the phosphorylated residues (Honda *et al.*, 1993). It was shown that Cdc25 protein dephosphorylated both the inhibitory phosphotyrosine and phosphothreonine residues of Cdc2. There are different isoforms of Cdc25 protein but only Cdc25C isoform is responsible for activation of Cyclin B/Cdc2 in *Xenopus* egg extracts (Hoffmann *et al.*, 1993; Nilsson and Hoffmann, 2000). Hereafter, I will refer to Cdc25C as simply Cdc25.

Once activated, Cyclin B/Cdc2 will then phosphorylate downstream substrates, driving the cell cycle from interphase into M-phase. Towards the end of M-phase, Cdc2 kinase is inactivated due to destruction of Cyclin B protein (Fig. 2; Murray *et al.*, 1989; Glotzer *et al.*, 1991; reviewed by Irniger, 2002). An N-terminally truncated Cyclin B (Δ90) protein can bind to and activate Cdc2, but is resistant to proteolysis. As a result, Cyclin B (Δ90) protein can maintain high Cdc2 kinase activity and arrest *Xenopus* egg extracts in M-phase (Murray *et al.*, 1989). The M-phase arrest induced by Cyclin B (Δ90) was not due to inhibition of proteolysis of endogenous Cyclin B protein which sustains the MPF activity. Since Cyclin B (Δ90) turns on the Cyclin degradation machinery, yet high levels of Cyclin B (Δ90)/Cdc2 activity are maintained, this M-phase arrest was more like an anaphase arrest. This was shown by the degradation of radiolabelled Cyclin B protein in a *Xenopus* egg extract that had been driven into M-phase by Cyclin B (Δ90) protein. Although the radiolabelled Cyclin B protein underwent degradation, the extract still contained high MPF activity due to nondegradable, radiolabelled Cyclin B (Δ90) (Murray *et al.*, 1989). These results showed that the degradation of Cyclin B was required for inactivation of Cdc2 kinase and that this degradation required the N-terminal region of Cyclin B protein. All mitotic Cyclin proteins contain a sequence of amino acids, referred to as the "destruction box" (Arg-X-X-Lys-X-X-X-X-Asn), located in the N-terminal region

(Glotzer *et al.*, 1991; reviewed by Irniger, 2002). Subsequently, it was shown that Cyclin B is degraded by the ubiquitin mediated proteolytic pathway, and that the N-terminal region of Cyclin B protein is necessary for degradation by this pathway. When a chimeric protein consisting of the N-terminal region of Cyclin B containing the destruction box fused to protein A was added to *Xenopus* egg extracts that had been driven into M-phase by Cyclin B (Δ90), the chimeric protein underwent ubiquitin-mediated proteolysis (Glotzer *et al.*, 1991). Thus, these results showed that Cyclin B/Cdc2 inactivation at the end of M-phase requires proteolysis of Cyclin B protein.

Although, activation of Cyclin B/Cdc2 kinase is required for entry into M-phase, the activation of other kinases is also required during progression of M-phase. Some of these kinases include polo like kinases (PLKs) (reviewed by Mayor *et al.*, 1999), aurora kinases (reviewed by Nigg, 2001), and mitogen activated protein kinases (MAPKs) (reviewed by Ferrell, 1999a; Masui, 2000). I will specifically focus on the MAPK pathway.

1.2 MAPK Activation and M-phase Arrest

Mitogen activated protein kinases (MAPK) comprise a family of ser/thr kinases that regulate many cellular functions, such as cell proliferation, motility, and differentiation (reviewed by Cobb, 1999). In general, the MAPK family consists of p42/44 Mitogen Activated Protein Kinases (p42/44MAPKs) or Extracellular signal Regulated Kinases (ERK2 and 1), p38 and Stress Activated Protein Kinase/Jun protein kinase (SAPK/JNK). The p42/44MAPKs are activated by a MAP kinase kinase called MAPK/ERK kinase (MEK), which phosphorylates tyrosine and threonine residues of p42/44MAPKs (reviewed by Cobb, 1999).

I will focus on the p42/44 MAPK pathway, specifically the p42 isoform (ERK2), since, to date, this is the isoform that has been most extensively characterized for its role in the regulation of the *Xenopus* cell cycle, (reviewed by Ferrell, 1999b). The p42MAPK pathway regulates many aspects of the cell cycle in *Xenopus*, such as maturation, M-phase arrest of the unfertilized egg, and the spindle assembly checkpoint.

1.2.1 MAPK Activation and M-phase Arrest *in vivo*

Masui and Markert (1971) described another activity present in unfertilized eggs called Cytostatic Factor (CSF). When the cytoplasm of unfertilized, mature eggs was microinjected into one cell of a two-cell stage embryo, the microinjected cell was arrested at metaphase with condensed chromosomes aligned on a spindle lacking asters (Masui and Markert, 1971; reviewed by Masui, 2001). CSF activity is naturally present in unfertilized, mature eggs and causes the eggs to arrest in metaphase of meiosis. Upon fertilization, an increase in intracellular Ca^{++} destabilizes CSF, and the egg is released from arrest. In 1989, Sagata and colleagues discovered that the ser/thr kinase Mos had similar characteristics to CSF activity. Mos protein was naturally present in unfertilized eggs and disappeared after fertilization just like CSF activity. When unfertilized and fertilized egg extracts were immunoprecipitated with Mos antibody and then immunoblotted with the same antibody, Mos protein was only present in unfertilized egg extracts. The microinjection of Mos mRNA into one cell of a two cell-stage embryo induced an M-phase arrest, with characteristics similar to CSF arrest (Sagata *et al.*, 1989). In addition, when Mos protein was immunodepleted from an unfertilized egg extract and then the extract was microinjected into one cell of a two cell-stage embryo, the recipient cell did not arrest at M-phase. This result showed that the immunodepleted unfertilized egg extract had lost CSF activity. Thus, these results showed that, in addition to having CSF activity itself, Mos must be a component required for CSF activity in M-phase-arrested, unfertilized eggs.

It was later found that Mos activates the MAPK pathway (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). Addition of Mos protein to *Xenopus* oocyte and egg extracts induced p42MAPK activation (Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993). Mos directly phosphorylated and activated the kinase upstream of p42MAPK, MEK *in vitro* (Posada *et al.*, 1993). Microinjection of thiophosphorylated p42MAPK into an embryo (Haccard *et al.*, 1993) induced a metaphase arrest, like the CSF arrest in unfertilized eggs. When MEK antibodies along with Mos protein were microinjected into one cell of a two-cell stage embryo, metaphase arrest was inhibited (Kosako *et al.*, 1994).

p90Ribosomal S6 kinase-1 (p90Rsk-1), a kinase that is a downstream target of p42MAPK, has been shown to induce CSF arrest (Bhatt and Ferrell, 1999; Gross *et al.*, 1999). Microinjection of a constitutively-active, mutant Rsk protein into one cell of a two-cell stage embryo caused a CSF arrest in the microinjected cell (Gross *et al.*, 1999).

In conclusion, CSF activity is naturally present in unfertilized *Xenopus* eggs before fertilization. Mos protein was shown to be a component of CSF arrest by activating the MAPK pathway. Furthermore, it has been shown that activation of p90Rsk in the p42MAPK pathway leads to CSF arrest in *Xenopus*. Therefore, activation of any component of the MAPK pathway leading to p90Rsk activation has been found to lead to CSF arrest.

1.2.2 MAPK Activation and M-phase Arrest of *Xenopus* Cycling Egg Extracts

As mentioned previously, concentrated extracts of *Xenopus* oocytes or eggs can be used as cell-free systems that reproduce cell cycle events *in vitro*, as they would normally occur *in vivo*. In 1983, Lohka and Masui developed the first cell-free system from amphibian eggs and showed that when demembranated sperm nuclei were added to extracts of eggs that had been artificially released from the M-phase arrest, chromosome decondensation, formation of a nuclear envelope, and DNA replication occurred, followed by recondensation of chromosomes. In addition, M-phase-arrested *Xenopus* egg extracts with sperm nuclei added, supported the direct formation of spindles (mainly monopolar spindles) (Lohka and Maller, 1985). In 1989, Murray and Kirschner modified the procedure for making this extract so that it could undergo multiple cell cycles *in vitro*. Therefore, cell-free systems have allowed studying many aspects of the cell cycle *in vitro* that would be difficult or impossible to study *in vivo*.

Activation of the MAPK pathway can induce an M-phase arrest *in vitro* in *Xenopus* cycling egg extracts, similar to CSF arrest *in vivo* (Minshull *et al.*, 1994; Walter *et al.*, 1997; Bitangcol *et al.*, 1998; Chau and Shibuya, 1998, 1999; Bhatt and Ferrell, 1999). Activation of p42MAPK during the increase of MPF activity by addition of Mos protein or constitutively-active MEK caused an M-phase arrest in

Xenopus cycling egg extracts. The M-phase arrest induced *in vitro* was similar to CSF arrest induced *in vivo*. It was characterized by a bipolar metaphase spindle and sustained, high levels of Cdc2 kinase activity (Minshull *et al.*, 1994; Abrieu *et al.*, 1996; Walter *et al.*, 1997; Chau and Shibuya, 1998, 1999). Cyclin B protein was stabilized in these M-phase-arrested extracts (Chau and Shibuya, 1998).

However, activation of the p42MAPK pathway can also sustain an M-phase arrest in *Xenopus* cycling egg extracts after Cyclin B/Cdc2 kinase inactivation and Cyclin B destruction (Chau and Shibuya, 1998; Guadagno and Ferrell, 1998; Bhatt and Ferrell, 1999). The addition of Mos protein leading to the activation of MAPK after the peak of MPF activity caused an M-phase arrest in cycling egg extracts with destabilized Cdc2 activity due to the destruction of Cyclin B protein (Chau and Shibuya, 1998; Guadagno and Ferrell, 1998). Surprisingly, nuclei incubated in these M-phase-arrested extracts maintained M-phase nuclear morphology (Chau and Shibuya, 1998; Guadagno and Ferrell, 1998), and M-phase specific phosphorylation of proteins was sustained (Chau and Shibuya, 1998). Using cycling egg extracts, p90Rsk has been shown to be the kinase downstream of p42MAPK required for M-phase arrest in *Xenopus* cycling egg extracts (Bhatt and Ferrell, 1999). When Rsk-2 protein was immunodepleted from a *Xenopus* cycling egg extract, addition of Mos protein to this extract could not induce an M-phase arrest. However, adding back Rsk-2 protein to this immunodepleted extract restored the ability of Mos to induce M-phase arrest. Cyclin B protein was proteolyzed and Cdc2 kinase was inactivated during this M-phase arrest. Thus, this result indicates that p90Rsk is the only kinase downstream of p42MAPK required for M-phase arrest.

In summary, if the p42MAPK pathway was activated during the increase of MPF (Cyclin B/Cdc2) activity, MPF activity was sustained with stabilized levels of Cyclin B protein. On the other hand, if the p42MAPK pathway was activated just after the peak of MPF activation, MPF activity levels fell and Cyclin B was degraded, yet the extract remained arrested in M-phase. Thus, different types of M-phase arrests can be obtained depending on timing of p42MAPK activation. Also, p90Rsk kinase is the only kinase downstream of p42MAPK required to induce either type of M-phase arrest in *Xenopus* cycling egg extracts. These results imply that although activation of

MPF is necessary for entry into M-phase, active p42MAPK is able to sustain M-phase even after the inactivation of MPF.

1.2.3 MAPK Activation and M-phase Arrest due to Spindle Assembly Checkpoint

The progression of the cell cycle is monitored by cell cycle checkpoints. Cell cycle checkpoints become activated when abnormal events such as DNA damage or improper spindle assembly occur in the cell, and inhibit progression of the cell cycle until the defect is repaired. One of these checkpoints is the spindle assembly checkpoint (SAC) which arrests the cell cycle at metaphase of M-phase in response to improper attachment of spindle microtubule fibers to chromosomes (reviewed by Hardwick, 1998).

The p42MAPK pathway is activated in the spindle assembly checkpoint in *Xenopus* (Minshull *et al.*, 1994; Takenaka *et al.*, 1997; Wang *et al.*, 1997; Cross and Smythe, 1998). The spindle assembly checkpoint (SAC) was activated by addition of nocodazole (a microtubule destabilizing drug) to a *Xenopus* egg extract with a high concentration of sperm nuclei (9,000/μl), causing an M-phase arrest with sustained p42MAPK activation. This SAC-induced M-phase arrest is different from CSF arrest in one important characteristic. Addition of $CaCl_2$ (that normally causes a release from CSF-induced M-phase arrest) to a SAC-induced M-phase-arrested *Xenopus* egg extract can not override the SAC-induced M-phase arrest (Minshull *et al.*, 1994). However, addition of MAPK phosphatase (MKP-1) or the MEK inhibitor, PD98059 (Alessi *et al.*, 1995; Dudley *et al.*, 1995; Cross and Smythe, 1998) to this extract caused a release from both the SAC-induced M-phase arrest and CSF-induced M-phase arrest (Minshull *et al.*, 1994). Furthermore, *Xenopus* extracts immunodepleted of p42MAPK were unable to arrest in M-phase when the SAC was induced (Takenaka *et al.*, 1997). The requirement for MAPK activation in M-phase arrest induced by SAC was also shown in *Xenopus* tadpole cells, where microinjection of *Xenopus* MAPK phosphatase (XCL100) into these cells caused a release from mitotic arrest induced by SAC (Wang *et al.*, 1997). It has not yet been shown if p90Rsk is the only kinase downstream of the p42MAPK pathway required for SAC-induced M-phase arrest.

In summary, the spindle assembly checkpoint (SAC) becomes activated in *Xenopus* cycling egg extracts in response to improper attachment of spindle fibers to chromosomes and induces an M-phase arrest by activation of the p42MAPK pathway. The activation of p42MAPK is required for both SAC and CSF, but interestingly, the M-phase arrests are different. One explanation for this difference was proposed by Minshull *et al.* (1994). In the SAC, the CaMKII pathway (which is normally activated by Ca^{++} when CSF arrest is overcome), is somehow inhibited, leading to the inhibition of a protein or a kinase downstream of the MAPK pathway that inhibits Cyclin B destruction and reinforces the induced M-phase arrest. In general, although these M-phase arrests are different, it seems p42MAPK is the downstream kinase that they both require for sustained M-phase arrest and its inactivation causes the release of these M-phase arrests.

1.3 Protein Phosphatase 1 (PP1) and its Regulation by Inhibitor-2 (Inh-2)

Phosphorylation and dephosphorylation of proteins in the cell is controlled by kinases and phosphatases, respectively. In general, there are more kinases than phosphatases in the cell. For example, in *Drosophila* there are an estimated 6 ser/thr kinases for every ser/thr phosphatase (reviewed by Cohen, 2002). Protein phosphatase 1 (PP1) is a ser/thr phosphatase which is member of a larger family of protein phosphatases (Antoniw and Cohen, 1976; reviewed by Cohen, 2002). PP1 isoforms identified so far include PP1α, β, and γ which share 90% identity in their amino acid sequences but exhibit different localization in mammalian cells (Andreassen *et al.*, 1998). The PP1 catalytic subunit is highly conserved among different species from yeast to mammals. *Xenopus* γ-PP1 subunit has already been cloned and it shares a very high degree of identity in amino acid sequence with PP1 subunits in other species (Lin *et al.*, 1999). Many inhibitors, such as okadaic acid and microcystin inhibit PP1, but they also inhibit other types of protein phosphatases, such as PP2A (reviewed by Dawson and Holmes, 1999).

To date, only a few catalytic subunits of protein phosphatases have been found in the cell. The catalytic subunits of PP1 are rarely found isolated from their regulatory subunits in the cell. The activity and subcellular localization of these

subunits are mainly regulated by their regulatory protein subunits. There are many regulatory subunits that have been reported (reviewed by Cohen, 2002). Some examples of these regulatory subunits of PP1 are nuclear inhibitor of PP1 (NIPP-1) (Van Eynde *et al.*, 1995), Inhibitor-1 (Huang and Glinsmann, 1976), DARPP-32 (Hemmings *et al.*, 1984) and Inhibitor-2 (Huang and Glinsmann, 1976).

Inhibitor-2 (Inh-2) is a heat stable, 23 kDa protein that is one of the regulatory subunits of PP1 which inhibits PP1 activity (Huang and Glinsmann, 1976; reviewed by Cohen, 2002). Inh-2 does not require phosphorylation to inhibit PP1 activity. On the other hand, phosphorylation of Thr 72 of Inh-2 by both glycogen synthase kinase-3 (GSK3) and MAPK *in vitro* has been shown to activate the PP1/Inh2 complex (Hemmings *et al.*, 1982; reviewed by DePaoli-Roach *et al.*, 1994). Casein Kinase II (CKII) phosphorylated serine residues of Inh-2 protein that increased GSK3 phosphorylation of Inh-2 *in vitro* leading to increased phosphatase activity (DePaoli-Roach, 1984). Sequence analysis showed that CKII phosphorylated Ser 86, 120 and 121 residues of Inh-2 (Holmes *et al.*, 1986; reviewed by DePaoli-Roach *et al.*, 1994). It has also been shown that p90Rsk phosphorylates serine residue(s) of Inh-2 *in vitro* (the precise residues were not determined), but this phosphorylation does not activate the Inh-2/PP1 complex (Wang *et al.*, 1995). The potential serine residues predicted to be phosphorylated by p90Rsk are not the same residues as those phosphorylated by CKII. The function of Inh-2 phosphorylation needs to be further investigated in relation to the cell cycle to determine if there are Inh-2 phosphorylations that would lead to inactivation of the activated complex. To date, all the phosphorylations that have been investigated have been shown to activate, enhance activation or have no effect on activation of the Inh-2/PP1 complex.

Inh-2 has been suggested to act as a chaperone, a protein that helps other proteins to fold into a correct conformation. Phosphorylation of Inh-2 by GSK3 seems to be required for the chaperone activity of Inh-2 protein (Alessi *et al.*, 1993; MacKintosh *et al.*, 1996). Phosphorylation of Inh-2 bound to denatured PP1 by GSK3 can activated the denatured PP1 (Alessi *et al.*, 1993). In addition, the expressed recombinant PP1 protein is less sensitive towards inhibitors such as microcystin, and regulatory subunit, Inhibtor-1, as compared to isolated native PP1 protein (Alessi *et*

al., 1993). When Inh-2 in the complex of Inh-2/expressed PP1 was phosphorylated by GSK3 it caused the complex to be more sensitive towards inhibitors such as microcystin (Alessi *et al.*, 1993). This result suggested that when Inh-2 was phosphorylated, the conformation of the complex was changed into one that perhaps was more similar to the *in vivo* state. Similar results were obtained using the *Drosophila* homologue of Inh-2 protein (Bennett *et al.*, 1999). It has not yet been determined if MAPK phosphorylation of the same threonine that leads to activation of the Inh-2/PP1 complex, would lead to the same chaperone effect.

Cdc2 kinase can phosphorylate the PP1 catalytic subunit in M-phase. Using site directed mutagenesis it was shown that threonine 320 of α-PP1 was phosphorylated by Cdc2 kinase and this phosphorylated PP1 was inactive (Dohadwala *et al.*, 1994). Similar results were obtained with Dis2 protein in fission yeast (Yamano *et al.*, 1994). A Dis2 protein in which threonine 316 was mutated to alanine (this threonine residue is homologous to threonine 320 in α-PP1) was not phosphorylated by active Cdc2 kinase *in vitro* and did not inactivate the phosphatase activity.

Interestingly, phosphorylations of Inh-2 and PP1 have also been shown to regulate localization of these two proteins in the cell. Using a phospho-specific antibody, the Cdc2-phosphorylated PP1 has been shown to be present in nucleus only at early stages (prophase) of M-phase in NIH3T3 cells (Kwon *et al.*, 1997). Mutation of the sites in Inh-2 phosphorylated by GSK3/MAPK and CKII (Thr 72, Ser 86 or Ser 120/121 to alanine) prevented accumulation of Inh-2 in the nucleus (Kakinoki *et al.*, 1997). However, it was not shown in this paper if localization of phosphorylated Inh-2 can cause a similar change in localization of the PP1 catalytic subunit. All of the previous work suggests that PP1 phosphatase activity and its localization may be regulated by the phosphorylation state of both the catalytic subunit and regulatory proteins.

Overall, Inh-2 protein is a regulatory subunit of PP1 that acts as a PP1 inhibitor or chaperone depending on its phosphorylation state. In addition, the above evidence suggests that phosphorylation of PP1 and Inh-2 can change the activity and localization of these proteins.

1.3.1 Role of PP1 and Inh-2 in Cell Cycle Regulation

PP1 regulates many cellular functions such as glycogen metabolism and cell cycle progression (reviewed by DePaoli-Roach *et al.*, 1994; reviewed by Zolnierowicz and Bollen, 2000). A number of observations have suggested that PP1 is involved in the regulation of exit from mitosis. In *Aspergillus nidulans*, a temperature-sensitive mutant of *bim*G11, which is homologous to PP1, caused an increase of condensed chromosomes and arrested the cells in M-phase at the restrictive temperature (43°C) (Doonan and Morris, 1989). In *Schizosaccharomyces pombe,* cold sensitive PP1 mutants (dis2) induced defects in chromosome separation at the restrictive temperature (20°C) (Ohkura *et al.*, 1988, 1989), and deletion of PP1 genes (sds21 and dis2) led to an M-phase arrest (Ishii *et al.*, 1996). A mutation of Glc7, a homologue of PP1 in *Saccharomyces cerevisiae* induced an M-phase arrest with undivided nuclei at the restrictive temperature (Hisamoto *et al.*, 1994). Similarly, mutation of PP1 87B (a homologue of PP1 in *Drosophila*) caused condensed chromosomes and M-phase arrest (Axton *et al.*, 1990). In higher eukaryotes, the microinjection of PP1 antibodies into rat embryo fibroblasts either during entry into or in mid-M-phase resulted in M-phase arrest as late as anaphase. On the other hand, the microinjection of PP1 catalytic subunit into rat embryo fibroblasts during late M-phase (anaphase) caused an early exit from M-phase as determined by an accelerated completion of cell division (Fernandez *et al.*, 1992). Overall, these reports provide evidence that PP1 activity is required for progression through and exit from M-phase of the cell cycle.

M-phase entry in *Xenopus* egg extracts results in increased phosphorylation of numerous proteins (e.g. Lohka *et al.*, 1987; Chau and Shibuya, 1998). This increased phosphorylation correlates with the decrease in the activity of protein phosphatases in M-phase. PP1 activity has been shown to oscillate during the cell cycle in *Xenopus* egg extracts with low activity during the increase in MPF activity but reaching higher activity during the peak of MPF activity and during S phase (Walker *et al.*, 1992). In order to examine PP1 activity, aliquots were collected from *Xenopus* egg extracts incubated with or without added recombinant Inh-2 protein for 10 min and then PP1 activity was measured by dephosphorylation of ^{32}P-radiolabelled histone H1. This result indicates that PP1 activity changes during M-phase with low activity during

entry into M-phase, but with increasing activity during mid- to late-M-phase. Although, inactivation of PP1 by Cdc2 phosphorylation alone does not explain the increasing activity of PP1 during mid- to late- M-phase, since PP1 activity is also regulated by its regulatory subunits, there may be other mechanisms that cause this increased activity. Due to the limits of these experiments, PP1 activity was not measured during the exit from M-phase and reentry into G1 phase, yet these results are consistent with previous reports that PP1 activity is inhibited during early M-phase and required during exit from M-phase.

Several lines of evidence suggest that Inh-2 protein may be required in regulation of cell cycle. Addition of Inh-2 protein (at concentration that inhibits PP1 activity in the extract) 20 min after release of M-phase arrest in unfertilized *Xenopus* egg extracts, induced early entry into M-phase as compared to control (Walker *et al.*, 1992). The entry into mitosis was monitored by M-phase morphology (nuclear envelope breakdown and chromosome condensation) and Cyclin B/Cdc2 kinase activity by measuring histone H1 phosphorylation. Inh-2 has also been shown to block DNA replication and overcome a DNA checkpoint arrest in *Xenopus* egg extracts (Walker *et al.*, 1992, Sohaskey and Ferrell, 1999). It was shown in these experiments that the amount of Inh-2 used caused PP1 inactivation. These results provide evidence for possible multiple functions of PP1 throughout the cell cycle.

The level of Inh-2 protein has been shown to undergo changes during cell cycle. The Inh-2 protein levels oscillated in rat fibroblasts with high expression during M and S-phases, shown by immunoblotting with anti-(human) Inh-2 antibody (Brautigan *et al.*, 1990). Furthermore, using Inh-2 protein fused with green fluorescent protein (GFP), Inh-2 localization in human fibroblast cells changed during the cell cycle with its accumulation in the nucleus during S-phase (Kakinoki *et al.*, 1997). Interestingly, localization of Inh-2 protein in the nucleus during S-phase is dependent on its phosphorylation by GSK3/MAPK and CKII, since mutation of these sites (Thr 72, Ser 86 or Ser 120/121) to alanine prevented accumulation of Inh-2 in the nucleus (Kakinoki *et al.*, 1997). Although, colocalization of Inh-2 and PP1 has not been examined in the cell, PP1 has been shown to localize in the nucleus and cytoplasm during interphase, and to the chromosomes as well as the cytoplasm during

M-phase (Anderassen *et al.*, 1998). Thus, the localization of phosphorylated Inh-2 in the nucleus could be required for both the activation and translocation of the associated PP1 catalytic subunit to the nucleus during S-phase.

Tournebize *et al.* (1997) have shown that Inh-2 protein is also involved in regulating microtubule length by inhibiting PP1 activity during cell cycle progression in *Xenopus* egg extracts. Addition of Inh-2 protein to *Xenopus* egg extracts affected microtubule length by causing the microtubules to remain short during the metaphase to anaphase transition in *Xenopus* egg extracts. When CaMKII was added to induce anaphase (exit from M-phase) in a CSF-arrested extract that was incubated with Inh-2 protein, the chromosomes did not separate (Tournebize *et al.*, 1997). Interestingly, PP1 inactivation had no effect on microtubule assembly during metaphase. When CSF-arrested extract was added to interphase extract to trigger the entry into M-phase with Inh-2 protein, the percentage of microtubules assembled was similar to that in the control extract (Tournebize *et al.*, 1997). This result is consistent with a requirement for PP1 activity during exit from M-phase and suggests a function for this activity in controlling microtubule length during late M-phase. Interestingly, addition of Inh-2 alone, or Inh-2 and Inh-1 together did not induce Cdc2 activation nor did their addition induce Cdc2 inactivation in *Xenopus* egg extracts (Felix *et al.*, 1990; Tournebize *et al.*, 1997). Furthermore, Inh-2 addition to *Xenopus* egg extracts did not delay the destruction of Cyclin B protein (Vorlaufer and Peters, 1998). In these experiments, Cyclin B destruction was monitored by instability of radioactive Cyclin B protein in the Cyclin B (Δ90)-induced M-phase arrest. This result is consistent with the Cyclin destruction machinery and Cdc2 kinase inactivation being independent of PP1 activity. Conversely, phosphorylation of PP1 by Cdc2 kinase causes PP1 inactivation (Dohadwala *et al.*, 1994; Yamano *et al.*, 1994). This result is consistent with the decrease of PP1 activity during increase of Cdc2 kinase activity seen in *Xenopus* egg extracts (Walker *et al.*, 1992). Thus, it can be hypothesized that oscillation of Cdc2 kinase activity is one way for regulating PP1 phosphatase activity during cell cycle.

Inh-2 protein has also been shown to be involved in maturation of *Xenopus* oocytes during meiosis (Foulkes and Maller, 1982). Microinjection of Inh-2 protein into *Xenopus* oocytes caused a delay in meiotic maturation after exposure to

progesterone. Although Walker *et al.* (1992) found that the addition of Inh-2 protein to *Xenopus* egg extracts caused an early entrance into M-phase, the results of Foulkes and Maller indicate that inactivation of PP1 by Inh-2 delays entry into M-phase of meiosis. The contradiction between the timing of entry into M-phase suggests that since PP1 activation/inactivation is required during other phases of the cell cycle, the different effects that are seen may depend on when PP1 becomes inactivated by Inh-2 protein.

In conclusion, PP1 activity is required for exit from M-phase, mutation of PP1 in these species inhibited exit from M-phase. PP1 phosphorylation by Cdc2 kinase leading to PP1 inactivation supports the notion that PP1 inactivation might be required early in M-phase, but during the latter part of M-phase PP1 activity is necessary for exit from M-phase. Inh-2 protein also regulates the cell cycle, and since it is the regulatory subunit of PP1, results obtained with Inh-2 suggest that most of its effects are likely due to its effects on PP1. Although, the function of PP1 activity during M-phase still needs to be further investigated, the Inh-2 protein studies have provided some possible clues into the function of PP1 activity in regulating microtubule length during M-phase, and its role during entry and exit from M-phase and in DNA replication. Further studies are still required to fully understand how PP1 activity precisely regulates cell cycle.

1.4 Proposal and Summary of Results

My research project focused on the role of Inh-2/PP1 in the regulation of the cell cycle in *Xenopus* cycling egg extracts. I have shown that the addition of Inh-2 protein to *Xenopus* cycling egg extracts caused an M-phase arrest of the cell cycle that was dependent on p42MAPK activation. This M-phase arrest was characterized by M-phase nuclear morphology (condensed chromosomes, an absence of nuclear envelopes), activation of the p42MAPK pathway, and sustained phosphorylation of M-phase specific phosphoproteins. However, the sustained M-phase arrest was not dependent on maintenance of high MPF activity, since Cyclin underwent degradation leading to a rapid drop in MPF activity. The M-phase arrest was dependent on p42MAPK activation, since inhibition of MEK, the direct activator of p42MAPK,

prevented M-phase arrest induced by Inh-2 protein. This result suggested that the M-phase arrest specifically required sustained activation of the MAPK pathway that was induced by inhibition of PP1, rather than a stabilization of M-phase due to a general inhibition of protein dephosphorylation.

Although the activation of the p42MAPK pathway was required for Inh-2-induced M-phase arrest, I hypothesized that activation of p90Rsk downstream of p42MAPK led to the phosphorylation of Inh-2, which was necessary for sustaining this arrest, in a potential feedback mechanism. Therefore, thiophosphorylated Inh-2 (that is resistant to dephosphorylation) could be used to test the ability of this modified Inh-2 to induce M-phase arrest. I found that activated p90Rsk can thiophosphorylate rabbit Inh-2 *in vitro*, and that the major site of phosphorylation is likely to be Ser 28. I have shown that the addition of the thiophosphorylated rabbit Inh-2 had the same effectiveness in inducing an M-phase arrest in *Xenopus* cycling egg extracts as unphosphorylated Inh-2. Moreover, human Inh-2, which lacks the putative major site of p90Rsk phosphorylation in rabbit Inh-2, was also able to induce an M-phase arrest with similar characteristics to that induced by rabbit Inh-2. Therefore, my results suggest that phosphorylation of Inh-2 by p90Rsk does not affect its ability to induce an M-phase arrest, and suggest that p90Rsk phosphorylation of Inh-2 is not involved in Inh-2-induced M-phase arrest.

CHAPTER 2:

Chapter 2: Materials and Methods

All of the reagents were purchased from Sigma or VWR unless otherwise indicated.

2.1 Preparation of Recombinant Histidine-tagged Inh-2 Protein

The cDNA encoding rabbit Inh-2 was subcloned into the pET28a plasmid (Maingat, unpublished) and expressed in *Escherichia coli* (*E. coli*). A colony of cells harbouring this construct was selected from a streaked plate and grown in 20 ml Luria-Bertani medium (LB) containing 50 μg/ml of kanamycin with constant shaking at 250 rpm for 25 hr at 37°C. The cells were pelleted by centrifugation for 6 min at room temperature using a swinging bucket rotor in a clinical centrifuge (top speed). The supernatant was discarded, then the pelleted cells were resuspended in 20 ml fresh LB and added to a 2 liter flask containing 500 ml of LB with 50 μg/ml of kanamycin. The flask was shaken for 2.5 hr at 250 rpm at 37°C until the $O.D._{600}$ reached 0.3. For the preinduction sample, 200 μl of bacterial culture from the flask was removed and centrifuged at 13,000xg for 2 min at room temperature using a fixed angle rotor in an Eppendorf microfuge to pellet the cells. The supernatant was removed and 50 μl of 1x sample buffer [2 % SDS (BDH), 80 mM Tris-HCl, pH 6.8, 10% volume per volume (v/v) glycerol, sufficient bromophenol blue (BDH) was added in order to monitor the sample on SDS-PAGE, 5% (v/v) β-mercaptoethanol] was added to the cells followed by boiling. The bacterial culture was then induced with 1 mM isopropylthio-β-D-galactoside (IPTG) and incubated under the same conditions for 4 hr. For the post induction sample, after 4 hr of induction, 100 μl of bacterial culture was removed from the flask, and the same procedure was followed as for the preinduction sample. After induction, the bacterial culture was centrifuged at 4,000xg for 10 min at 4°C using a GSA rotor in a Sorvall high speed centrifuge. The pelleted cells were resuspended in lysis buffer (125 mM NaCl, 1.0% Tween-20, 10 μg/ml each of leupeptin, chymostatin, and pepstatin, 2.0 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5) on ice, then the cells were frozen (liquid nitrogen) and thawed twice. Next, the cells were lysed by sonication of the cell suspension on ice (3x 20 pulse sets at 6 output, 30% duty, separated by 1 min using the broad probe). Then, the sonicate was centrifuged at 20,000xg for 30 min at 4°C using a fixed angle,

SS-34 rotor in a Sorvall high speed centrifuge. The supernatant (pre-binding sample) was removed and added to Ni^{2+}-charged nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) for 1 hr and mixed end-over-end at 4°C. The pre and post-binding samples were prepared by removing 10 µl of prep before (pre) and after (post) binding to the beads. The samples were then added to 10 µl of 2x sample buffer for gel analysis. After binding, the beads underwent a series of washes (each end-over-end for 10 min at 4°C). The washes were 3 times with lysis buffer (without protease inhibitors) with 1.0% Tween-20, once with lysis buffer with 1.0% Tween-20 and 0.5 M NaCl, and twice with lysis buffer with 1.0% Tween-20. The next series of washes were done at room temperature: 3 times with lysis buffer and twice with final wash buffer (125 mM NaCl, 20 mM imidazole, 25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5).

The His-tagged Inh-2 protein was then eluted with increasing concentrations of imidazole (50 to 250 mM) in dilution buffer (125 mM NaCl, 25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5). The first elution was done using 50 mM imidazole and two elutions were done for each imidazole concentration. For gel analysis, 6 µl of each eluate was added to 6 µl of 2x sample buffer. The pre-, post-binding samples and eluates were analyzed on a 17.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (acrylamide/bis, 241:1), then the gel was stained with Coomassie blue, destained and dried. The eluted fractions that contained His-tagged Inh-2 protein were pooled and dialyzed against dialysis buffer [5 mM $MgCl_2$, 20% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-KOH, pH 7.0] at 4°C, using 3,500 molecular weight (M.W.) cut-off dialysis tubing (Fisher Scientific). The protein was then concentrated by dialysis against the same buffer containing 10% polyethylene glycol (M.W. 20,000) at 4°C. The protein concentration was determined at the end by analyzing the samples along with different amounts of bovine serum albumin (BSA) on a 17.5% SDS-polyacrylamide gel (acrylamide/bis, 241:1). The protein concentration was measured using the ImageQuant 5.1 program.

The PP1 proteins were a gift from Dr. C. Holmes. The cDNA encoding human γ-PP1 was subcloned into the pCW vector and the protein was expressed in *E. coli*. The PP1 mutants R308A and T311D were generated by site directed mutagenesis.

2.2 Preparation of *Xenopus* Egg Extracts

2.2.1 Induction of Ovulation

Female *Xenopus* frogs were first injected (in the dorsal lymph sac) with 100 I.U. of pregnant mare's serum (PMS; Follicle Stimulating Hormone activity) two to three days before the day of the experiment followed by injection of 1000 I.U. of human chorionic gonadotropin (HCG; Luteinizing Hormone activity) 12-14 hr before the start of the dejellying. After the injection with HCG, female frogs were kept in 0.1 M NaCl and eggs were collected (spawned eggs and eggs obtained by gently squeezing the female frog).

2.2.2 Dejellying Eggs

Interphase-arrested, M-phase-arrested and cycling *Xenopus* egg extracts were made using dejellied, unfertilized *Xenopus* eggs (Murray, 1991; Bitangcol *et al.*, 1998). The eggs were dejellied in 0.02 g/ml of L-cysteine (hydrochloride) dissolved in 0.16 M of NaOH solution with a final pH between 8.1-8.3. The dejellied eggs were then washed at least 10 times with egg washing solution [0.1 M NaCl, 0.01 M HEPES-NaOH, pH 7.0] and examined under the dissection microscope to remove any activated (see section 2.2.3) or degenerated eggs.

2.2.3 Cycling Egg Extract

For cycling egg extracts, dejellied eggs were electrically activated (2 one second shocks of 15 volts AC, separated by 5 seconds) in 20% Steinberg's solution [11.6 mM NaCl, 0.134 mM KCl, 0.08 mM $MgSO_4.7H_2O$, 0.68 mM $Ca(NO_3)_2.4H_2O$, 0.92 mM Tris(hydroxyamino) methane, 0.8 mM HCl, 0.03 mg/ml penicillin G, 0.05 mg/ml streptomycin sulfate, 0.1 mg/ml sulfathiazole, pH 7.4]. The activation of eggs was monitored by contraction of the animal hemisphere (brown pigment) of eggs. This contraction occurred approximately between 3-10 min after activation of the eggs. Just prior to making the extract, the activated eggs were washed 3 times in extraction buffer (50 mM Sucrose, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10

mM HEPES-KOH, pH 7.7) and twice with the same extraction buffer that contained protease inhibitors (10 μg/ml each of pepstatin, leupeptin, and chymostatin).

The activated eggs were transferred to a 3 ml Ultraclear centrifuge tube (Beckman) containing 0.6 ml of silicon oil (Versalube F-50) overlaid with 0.6 ml of extraction buffer with protease inhibitors and 100 μg/ml of cytochalasin B [added from a stock solution of 10 mg/ml in dimethyl sulfoxide (DMSO), stored at -20°C]. The eggs were packed by centrifugation at 1,060xg for 1 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. Then, the excess oil and buffer were aspirated. The packed eggs were then crushed by centrifugation (25-35 min after activation) at 10,200xg for 15 min at 4°C using the same rotor, and the cytoplasmic layer was transferred to an ice-chilled 1.6 ml microfuge tube before recentrifugation at 10,200xg for 15 min at 4°C using the same rotor and centrifuge.

After the last centrifugation, the cleared cytoplasm was removed and 1 volume of an ATP-regenerating system [40 mM Adenosine triphosphate (ATP)/Mg^{2+} (Roche), 0.4 M creatine phosphate (Roche), 2 mg/ml creatine kinase (Roche)] was mixed with 37 volumes of the extract. Demembranated sperm nuclei were added to a final concentration of 75-100 nuclei/μl of extract. A schematic diagram of this procedure is shown in Figure 3 (Murray, 1991; Bitangcol *et al.*, 1998).

2.2.4 Interphase-Arrested Egg Extracts

For interphase-arrested, activated egg extracts, unfertilized eggs were dejellied and washed in the same way as for cycling egg extracts as described above (see section 2.2.3). The dejellied eggs were incubated in 20% Steinberg's solution with 100 μg/ml of cycloheximide for 7-10 min and then electrically activated (see section 2.2.3) in the same buffer and washed 3 times in extraction buffer with 100 μg/ml of cycloheximide and twice with the same buffer that contained protease inhibitors (see section 2.2.3). The activated eggs were crushed at 32-37 min after activation and the same centrifugation conditions were used to obtain this extract. The same ratio of ATP-regenerating system was added to the extract, which was used either immediately or was frozen in liquid nitrogen in aliquots of 50 μl and stored at -80°C.

2.2.5 M-phase-Arrested Egg Extracts

For M-phase-arrested, unfertilized egg extracts, dejellied eggs were washed 3 times in a CSF extraction buffer [50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid (EGTA), 20 mM NaF, 10 mM HEPES-KOH, pH 7.7] and twice with the same buffer that contained protease inhibitors (as described above, see section 2.2.3). The egg extracts were made in the same way as described above (see section 2.2.3) with the exception that cytochalasin B was added to extract after the first centrifugation to a final concentration of 50 μg/ml.

2.3 Cycling Egg Extract Assays

For cycling *Xenopus* egg extract assays, reactions were made by aliquotting cycling egg extract that contained ATP-regenerating system and demembranated sperm into a 1.6 ml microfuge tube on ice (Bitangcol *et al.*, 1998). Rabbit Inh-2 protein (either Calbiochem or His-tagged Inh-2), human Inh-2 (Gift from Dr. C. Holmes), or buffer [for rabbit (Calbiochem) and human Inh-2 (see section 2.6.3.1), and for His-tagged Inh-2 (see section 2.1)] was added to 5%-14% (v/v) of the reaction (the actual additions are specified in the legends). The final volume of each reaction was 70 μl. After taking an initial set of samples, the reactions were then incubated in a water bath at 21-23°C. Immunoblot, histone H1 kinase (H1K), and cytology samples were taken every 10 min for analysis. The samples were taken by removing 2 μl of reaction and mixing it with 20 μl of ice-chilled H1 kinase buffer (50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 10 mM NaF, 5 mM EGTA, 10 mM HEPES-KOH, pH 7.7). After mixing, 17 μl of the mixture was added to 20 μl of 2x sample buffer (Laemmli, 1970) containing 10 mM NaF and 1 mM Na_3VO_4, boiled for 5 min and the remaining 5 μl was immediately frozen in liquid nitrogen. For cytology samples, 2.5 μl of reaction was removed using a wide-orifice pipette tip and mixed gently with 10 μl of EGS fixative (see section 2.8). At the end of the assay, the immunoblot and H1K samples were stored at -80°C. The cytology samples were stored at 4°C.

The PD98059 drug (Calbiochem) was prepared as a stock solution of 50 mM in DMSO and stored at -20°C. It was added to reactions to a final concentration of 300 μM (1: 167 dilution).

2.4 Antibodies and Immunoblotting

For immunoblot analysis (Bitangcol *et al.*, 1998), samples were analyzed on 12.5% SDS-polyacrylamide gels (acrylamide/bis, 120:1), except for human Inh-2 antibody immunoblots, in which samples were separated on 17.5% gels. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P filter, Millipore) at 4°C using a Hoeffer immunoblotting apparatus at 100 V for 2.5 hr or overnight at 50 mAmp. The membranes were incubated with primary antibody overnight at room temperature. The primary antibodies were: affinity-purified Cdc25C antibodies (Bitangcol *et al.*, 1998), phosphotyrosine antibodies (Druker *et al.*, 1989; Bitangcol *et al.*, 1998), p90Rsk-1 antibodies (Bhatt and Ferrell, 1999; product SC-231, Santa Cruz Biotechnology), Cyclin B2 antibodies (van der Velden and Lohka, 1993; Bitangcol *et al.*, 1998), MPM-2 antibodies (Chau and Shibuya, 1998; Upstate Biotechnology Incorporated, Cat # 05-368), Human Inh-2 antibodies (Gift of Dr. D. Brautigan) and phospho-p44/42 antibodies (New England Biotechnology, Cat # 9101). Goat anti-rabbit or anti-mouse alkaline phosphatase-conjugated secondary antibodies (anti-rabbit; Cat. # A3687; anti-mouse; Cat. # A3562) were used at 1:3000 dilution for 1 hr at room temperature to detect the primary antibodies. Immunoblots were developed using the substrates 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) according to the manufacturer's instructions (Promega, Cat. # P3791).

2.5 Immunoprecipitation

2.5.1 p90Rsk-1

Either a freshly made or thawed extracts of interphase-arrested, activated eggs (see section 2.2.4) or M-phase-arrested, unfertilized eggs (see section 2.2.5) were centrifuged at 10,200xg for 5 min at 4°C using a swinging bucket rotor in an

Eppendorf 5417R microfuge. The supernatant was removed and transferred to a new 1.6 ml microfuge tube. For M-phase-arrested extracts, 1 volume of ATP-regenerating system [40 mM ATP/Mg^{2+} (Roche), 0.4 M creatine phosphate (Roche), 2 mg/ml creatine kinase (Roche)] was added to 37 volumes of extract. Then NaF was added to 20 mM (1:100 dilution) from a stock of 1 M.

For 450 µl of either extract, 45 µl of p90Rsk-1 antibodies were added. The mixture was incubated on ice for 60 min, then the mixture was added to a fresh tube that contained 45 µl of protein A-Sepharose CL-4B beads and Sepharose CL-4B [1:1, v/v (Pharmacia Biotechnology Incorporated)], followed by mixing end-over-end for 1 hr at 4°C. The mixture was then centrifuged at 10,200xg for 5 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge, followed by removal of the supernatant. The pelleted beads were washed end-over-end for 10 min at 4°C. They were first washed with CSF extraction buffer (see section 2.2.5) that contained 1.0% Tween-20, followed by wash buffer containing 1.0% Tween-20 and 1 M NaCl, then wash buffer containing 1.0% Tween-20 and twice with wash buffer. At the end, the pelleted beads were washed once with kinase buffer (1 mM β-mercaptoethanol, 5 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.0) and resuspended in 45 µl kinase buffer. The resuspended immunoprecipitates were then aliquotted (10 µl slurry of 1 volume beads and 1 volume kinase buffer). The aliquots were frozen in liquid nitrogen, and stored at -80°C.

2.5.2 Cyclin B2/Cdc2

Either freshly-made, or thawed M-phase-arrested, unfertilized egg extracts were centrifuged at 10,200xg for 5 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. The supernatant was removed and transferred to a new 0.6 ml microfuge tube. 40 µl of supernatant was mixed with 160 µl of dilution buffer (80 mM NaCl, 20 mM EGTA, 10 mM $MgCl_2$, 10 mM NaF, 10mM HEPES, pH 7.4), then 4 µl of Cyclin B2 antibody (Gift from Dr. J. Maller) was added. The mixture was incubated on ice for 90 min, and was then added to a fresh tube that contained 20 µl of protein G–Sepharose CL-4B beads (Pharmacia Biotechnology Incorporated) and mixed end-over-end for 1 hr at 4°C. The mixture was then centrifuged at 10,200xg for

5 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge, followed by removal of the supernatant. The pelleted beads were washed end-over-end for 10 min at 4°C, centrifuged as described above, and the wash buffer was removed at the end of each wash. Beads were washed once with dilution buffer containing 0.5% Tween-20 followed by centrifugation, as described above. The pelleted beads were then washed twice with dilution buffer with 0.5% Tween-20 and 0.5 M NaCl, followed by one wash with dilution buffer with 0.5% Tween-20, and a final wash with dilution buffer. Then 1 bead bed volume of dilution buffer was added and the immunoprecipitates were aliquotted (5 µl), frozen in liquid nitrogen and stored at -80°C.

2.6 Kinase Assays

2.6.1 Histone H1 Phosphorylation Assay

Histone H1 phosphorylation assays for *Xenopus* cycling egg extract reactions (Bitangcol *et al.*, 1998) were done by first rapidly thawing the H1K samples (5 µl) followed by centrifugation at 10,200xg for a few sec at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. Then, 3 µl of kinase mixture [10 mM $MgCl_2$, 5 mM EGTA, 0.2 mg/ml histone H1 (Boehringer Mannheim), 60 µM unlabeled ATP, 0.5 µCi/µl γ-^{32}P-ATP (NEN), 20 µM protein kinase A inhibitor peptide (PKI), 10 µM calmidazolium, and 20 mM HEPES-NaOH, pH 7.5] was added to each sample, and reactions were incubated for 20 min at 21-23°C. After 20 min, reactions were quenched with 8 µl of 2x sample buffer. Samples were then analyzed on a 15% SDS-polyacrylamide gel (acrylamide/bis, 174:1) and the gel was then processed for autoradiography. For quantitation of ^{32}P incorporation, the gels were analyzed by phosphorimager (see section 2.7).

Histone H1 phosphorylation kinase assays for Cyclin B2/Cdc2 immunoprecipitates were performed and analyzed exactly as above except the kinase mixture consisted of [10 mM $MgCl_2$, 5 mM EGTA, 0.2 mg/ml histone H1 (Boehringer Mannheim), 60 µM unlabeled ATP, 0.5 µ Ci/µl γ-^{32}P-ATP (NEN), and 20 mM HEPES-NaOH, pH 7.5] and the tubes were mixed during the incubation.

2.6.2 PP1 Phosphorylation Assays

PP1 kinase assays were done similarly to the histone H1 kinase assays. The Cyclin B2/Cdc2 immunoprecipitate samples (5 µl) were used for the PP1 kinase assays. After thawing the samples on ice, 10 µl of kinase mixture [5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 60 µM unlabeled ATP, 1.2 µM PP1 proteins (Gift from Dr. C. Holmes), 0.5 µCi/µl γ-^{32}P-ATP, and 20 mM HEPES-KOH, pH 7.0] was added to each sample, and reactions were incubated for 1 to 1.5 hr at 21-25°C. At the end of incubation, the reactions were centrifuged at 11,700xg for 10 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. Then, the supernatant (soluble portion) was removed from the pellet (beads). The pellets were mixed with 20 µl of 2x sample buffer and boiled. The pellets were then separated on a 15% SDS-polyacrylamide gel and the gel was then processed for autoradiography. For quantitation of ^{32}P incorporation, the gels were analyzed by phosphorimager (see section 2.7).

2.6.3 Inh-2, S6 peptide, and Egg Extract Supernatant Phosphorylation Assays

2.6.3.1 Inh-2 Phosphorylation Assays

The p90Rsk-1 immunoprecipitates from an extract of interphase-arrested, activated eggs or an extract of M-phase-arrested, unfertilized eggs were used in an *in vitro* kinase assay (Wang *et al.*, 1995). The suspension (10 µl) of p90Rsk-1 was thawed and 10 µl of kinase mixture [5 mM $MgCl_2$, 1mM β-mercaptoethanol, 0.1 mg/ml rabbit Inh-2 (Calbiochem), 60 µM unlabeled ATP, 0.5 µCi/µl γ-^{32}P-ATP (NEN), and 20 mM HEPES-NaOH, pH 7.0] was added to each reaction and they were incubated for 60 min at 21-22°C. At the end, the reactions were quenched with 2x sample buffer. Samples were then separated on a 17.5% SDS-polyacrylamide gel and processed for autoradiography.

For thiophosphorylation of Inh-2 protein, an *in vitro* kinase assay was done by adding 12 µl of kinase mixture [40 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 mM unlabeled γ-S-ATP, 2 µCi/µl γ^{35}S-ATP (ICN)] to the

p90Rsk-1 immunoprecipitated from M-phase-arrested, unfertilized eggs (active p90Rsk-1). As a substrate, 0.37-0.56 μg/μl of rabbit Inh-2 protein (Calbiochem), or buffer [50 mM Tris-HCl, pH 7.0, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.01% Brij-35 and 50% glycerol], or 0.37-0.56 μg/μl human Inh-2 protein (Gift from Dr. C. Holmes) in buffer [0.1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM ammonium acetate, pH 5.0, which was brought to pH 7.0 by adding 1 M Tris solution] were added to the kinase reaction. The kinase reactions were mixed end-over-end for 8-25 hr at 21-26°C. The kinase reactions were then centrifuged twice at 11,700xg for 10 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. The supernatant was removed after the last spin and was added to a mini-column (Wizard kit, Promega) that contained 125-200 μl of 0.1 g/ml slurry of G-25 superfine Sephadex (Pharmacia Biotechnology Incorporated) in the kinase buffer (5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 20 mM HEPES-KOH, pH 7.0), the column had been washed twice with the kinase buffer prior to addition of the supernatant. After the addition of the supernatant, the column was centrifuged at 5,000xg for 30 sec at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge and the eluate was collected in the receiving tube. This procedure was done twice for each supernatant. Samples from each supernatant were mixed with 1x sample buffer, separated on a 17.5% SDS-polyacrylamide gel and processed for autoradiography to verify phosphorylation of Inh-2. The collected eluates were stored at -20°C until they were used for cycling *Xenopus* egg extract assays.

2.6.3.2 S6 Peptide Phosphorylation Assays

For the kinase reactions with S6 peptide, the p90Rsk-1 immunoprecipitates from an extract of interphase-arrested, activated eggs and an extract of M-phase-arrested, unfertilized eggs (see section 2.2.5) were used in an *in vitro* kinase assay (Erikson and Maller, 1985). The suspension (10 μl) of p90Rsk-1 was thawed and 10 μl of kinase mixture [5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mg/ml of human 40S ribosomal protein S6 peptide, amino acids 231-239 (Santa Cruz Biotechnology), 60 μM unlabeled ATP, 0.5 μCi/μl γ-^{32}P-ATP (NEN), 5 μM of microcystin (Gift of Dr. C. Holmes) and 20 mM HEPES-NaOH, pH 7.0] was added to each reaction and they

were incubated for 35 min at 21-22°C. The reactions were quenched with 10 µl of stop buffer (10 mM EDTA, 100 mM NaF, 100 mM $Na_4P_2O_7$, 10 mM Na_3VO_4) centrifuged, and the supernatant was removed. For scintillation counting, 10 µl of supernatant was spotted onto a half-inch square of P81 (phosphocellulose) paper and dried. The P81 squares were washed 5 times with 0.5% phosphoric acid and then dried. Each square was put in a separate vial with 5 ml of scintillation liquid (Labindustries Incorporated) and counted using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman).

2.6.3.3 Boiled Egg Extract Supernatant Phosphorylation Assay

For the kinase reaction with supernatant of boiled *Xenopus* cycling egg extract (Inh-2 protein remains soluble after boiling), the p90Rsk-1 immunoprecipitates from an extract of interphase-arrested, activated eggs and an extract of M-phase-arrested, unfertilized eggs (see section 2.2.5) were used in an *in vitro* kinase assay (Erikson and Maller, 1985). The Cyclin B2/Cdc2 immunoprecipitates (IP) from an extract of M-phase-arrested, unfertilized eggs (M) were also used (see section 2.5.2). A *Xenopus* cycling egg extract (see section 2.2.3) was prepared. The extract was boiled for 5 min, centrifuged at 11,700xg for 50 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. The supernatant (soluble portion) was removed from the pellet. The kinase reactions were done in presence of γ^{32}P-ATP and the supernatant of boiled cycling egg extract. The reactions were incubated for 2 hr at 23-24°C. At the end of incubation, the reactions were centrifuged at 11,700xg for 15 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge and the supernatant (soluble portion) was removed from the pellet (beads). The supernatant samples were separated on a 17.5% SDS-polyacrylamide gel, stained with Coomassie blue, destained and then dried. The human-Inh-2 antibody was used to immunoprecipitate Inh-2 protein from the supernatants (similar procedure to p90Rsk-1 immunoprecipitation, see section 2.5.1). Then, the immunoprecipitated samples were separated on a 17.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, destained and then dried.

2.7 Autoradiography and Phosphorimager Analysis

For autoradiography, the dried gels were placed in a cassette over X-OMAT film (Kodak) for 1 to 24 hr. The film was developed using the Kodak X-OMAT M35 processor (Kodak). The quantitation of ^{32}P and ^{35}S incorporation was determined by phosphorimager analysis. The dried gels were placed on a phosphorimager plate for 10 to 24 hr, processed by phosphorimager (Typhoon 8600) and quantified using the ImageQuant 5.1 program.

2.8 Cytology and Fluorescence/Phase Contrast Microscopy

The EGS fixative was prepared [1 volume of 0.2 M ethylene glycol-bis (succinic acid *N*-hydrosysuccinimide ester)(EGS) dissolved in DMSO and centrifuged at 13,000xg for 5 min at room temperature using a fixed angle rotor in an Eppendorf microfuge; 24 volumes of 100 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 20 mM HEPES-KOH, pH 7.5 and 2 µg/ml Hoechst 33342 (Roche)] (Bitangcol *et al.*, 1998) and was centrifuged at 13,000xg for 5 min at room temperature using a fixed angle rotor in an Eppendorf microfuge.

The cytology samples were then examined by mounting 8.5 µl of fixed samples on a microscope slide using a wide-orifice pipette tip. Each sample was covered with a 22 mm square cover slip and sealed with nail polish. The samples were then examined using a Zeiss light/fluorescence microscope under 40x or 100x oil immersion objectives. Photographs were taken with a microscope camera (Zeiss-MC100) using Elite chroma 100 slide film (Kodak). The slides were then scanned and saved as Tiff files.

2.9 Inh-2 Amino Acid Sequence Comparison

The rabbit and the human Inh-2 protein amino acid sequences were compared with the predicted *Xenopus* Inh-2 partial amino acid sequence. The partial nucleotide sequence of *Xenopus* Inh-2 (Genbank accession number: BI444584) was translated using the ORF Finder (Open Reading Frame Finder, NCBI) program. Then, the predicted *Xenopus* Inh-2 amino acid sequence was compared to the rabbit (Genbank accession number: M95581), and the human Inh-2 (SWISS-PROT accession number:

P41236) protein amino acid sequences using ClustalW. (EMBnet.ch.org) program.

CHAPTER 3:

Chapter 3: Results

3.1 *Xenopus* Cycling Egg Extract Assays

The *Xenopus* cycling egg extract system is a well-established system that allows studying cell cycle events *in vitro*, as they would occur *in vivo* (reviewed in section 1.1.1, Lohka and Masui, 1983; Murray and Kirschner, 1989). One of the advantages of this system is that large quantities of extract can be obtained for biochemical analysis and cell-free systems (e.g. Lohka *et al.*, 1988). To study cell cycle events *in vitro,* extracts were made from activated *Xenopus* eggs, then an ATP-regenerating system and demembranated sperm nuclei were added to the extract (see section 2.2, Fig. 3). Then, assays were carried out at 21 to 23°C, and samples were taken from each reaction at 10 min intervals for 2 hr for analysis of cell cycle progression. The nuclei incubated in the extract respond to changes in cell cycle phase *in vitro*, as they would *in vivo*. In addition, several biochemical parameters were used to monitor the progression of the cell cycle in the cycling egg extracts.

One parameter used to monitor cell cycle progression *in vitro* was the change in morphology of the sperm nuclei during incubation of the reactions. Initially, the sperm chromatin decondensed and a nuclear envelope was assembled from components in the egg extract (interphase), then nuclear envelope breakdown (NEBD) and chromosome condensation (CC) were induced upon entry of the extract into M-phase. To monitor NEBD and CC, samples from each reaction were taken during the incubation, fixed, stained with a fluorescent, DNA-binding dye (Hoechst) and then examined using fluorescence and phase/contrast microscopy.

MPF (Cyclin B/Cdc2) kinase activity was another parameter that was measured in these assays. Cyclin B/Cdc2 kinase activation is required for entry into M-phase (reviewed in sections 1.1.2). Cdc2 kinase activity was determined by using an *in vitro* kinase assay with histone H1 protein as an exogenous substrate (Lohka *et al.*, 1988). In addition, since Cdc2 kinase activity is dependent on association with Cyclin B (reviewed by Irniger, 2002) levels of Cyclin B protein were also determined by immunoblotting with anti-Cyclin B2 antibody.

The third parameter that was determined was activation of the MAPK pathway. The p42MAPK protein becomes activated upon phosphorylation by MEK protein (reviewed by Cobb, 1999). p42MAPK is phosphorylated on threonine and tyrosine residues (T-E-Y sequence) leading to its activation (Anderson *et al.*, 1990; Payne *et al.*, 1991). Its sustained activation at the appropriate time in the cell cycle leads to an M-phase arrest (reviewed in section 1.2). To monitor p42MAPK activation, the reaction samples were immunoblotted using anti-phosphotyrosine antibody or anti-phospho-MAPK antibody. p90Rsk is the target downstream of p42MAPK necessary for the induction of M-phase arrest *in vitro,* in *Xenopus* cycling egg extracts (Bhatt and Ferrell, 1999) and *in vivo* (Gross *et al.*, 1999, 2000). Activation of p90Rsk is due to phosphorylation and was determined by immunoblotting with anti-p90Rsk-1 antibody to detect the slower mobility, hyperphosphorylated form of the kinase (e.g. FIG. 9, reviewed by Frodin and Gammeltoft, 1999).

Another parameter that was monitored was phosphorylation of M-phase specific phosphoproteins, specifically Cdc25 protein during the incubation period. Cdc25 protein becomes hyperphosphorylated during entry into M-phase (Kumagai and Dunphy, 1992). Hyperphosphorylation of Cdc25 protein is used as a marker for M-phase (e.g. Bitangcol *et al.*, 1998). This hyperphosphorylation was monitored by immunoblotting the reaction samples with anti-Cdc25 antibody (to detect the slower mobility form). To monitor the overall phosphorylation level of M-phase specific phosphoproteins, anti-MPM-2 antibody was used. This monoclonal antibody only recognizes proteins when they become phosphorylated specifically during M-phase (Davis *et al.*, 1983).

In summary, the parameters used to monitor the progression of cell cycle in *Xenopus* cycling egg extract assays were: changes in nuclear morphology, Cyclin B/Cdc2 kinase activity, activation of the MAPK pathway, and phosphorylation of M-phase specific phosphoproteins.

3.2 Addition of Rabbit Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extracts

A recombinant His-tagged Inh-2 protein was expressed in *E. coli*, purified, and then concentrated to 1-2 mg/ml (Fig. 4). Rabbit Inh-2 protein consists of 205 amino acids with expected molecular weight of 23 kDa that runs at a molecular weight of around 31 kDa on SDS-polyacrylamide gels (Foulkes and Cohen, 1980; Park *et al.*, 1994). For the experiments involving the addition of rabbit Inh-2 protein to *Xenopus* cycling egg extracts, both His-tagged Inh-2 protein and a commercially available recombinant Inh-2 protein (Calbiochem) were used. Both rabbit Inh-2 protein preparations gave similar results.

Each reaction was made by adding the same volume (relative to the total of the final reaction) of either buffer or rabbit Inh-2 protein to an aliquot of *Xenopus* cycling egg extract containing demembranated sperm nuclei (Fig. 5). The additions were made on ice before the incubation of the reactions at 21-23°C and samples were taken for analysis every 10 min for 2 hr. The gel samples were immunoblotted with anti-Cdc25 antibody (Fig. 5A, top panels), anti-phosphotyrosine antibody (second panels), anti-p90Rsk-1 antibody (third panels), and anti-MPM-2 antibody (bottom panels). The nuclei in these reactions were monitored for M-phase morphology (NEBD and CC), indicated by bars below the Cdc25 immunoblot in Fig. 5A.

The reaction to which buffer was added underwent two cell cycles as indicated by entry into M-phase at 50 and 100 min. M-phase was characterized by Cdc25 hyperphosphorylation, M-phase nuclear morphology (NEBD and CC), and M-phase specific phosphorylation of proteins at these time points (Fig. 5A, top and bottom left panels). p42MAPK was transiently phosphorylated with low levels of tyrosine phosphorylation at 50 min (Fig. 5A, second left panel). This transient activation of p42MAPK has been seen in work from our laboratory and others (Minshull *et al.*, 1994; Bitangcol *et al.*, 1998; Guadagno and Ferrell, 1998). The p90Rsk-1 hyperphosphorylation was not detected in this reaction (Fig. 5A, third left panel).

In contrast, the reaction to which rabbit Inh-2 protein was added entered M-phase at the same time as the control reaction, but was arrested in M-phase as characterized by sustained hyperphosphorylation of Cdc25, and M-phase nuclear

morphology (Fig. 5A, top right panel). The p42MAPK pathway is activated in the Inh-2-induced M-phase arrest as shown by p42MAPK tyrosine phosphorylation and hyperphosphorylation of p90Rsk-1 (Fig. 5A, second, third right panels). Finally, there is a sustained phosphorylation of M-phase specific phosphoproteins as indicated by the MPM-2 immunoblot (Fig. 5A, bottom right panel).

Fig. 5B (left pair of images) shows the nuclear morphology at the 120 min time point of a reaction to which buffer was added. The Hoechst staining shows decondensed chromatin and phase contrast shows a nuclear envelope at this time point indicating that the reaction is in interphase. The right pair of images (Fig. 5B) shows the cytology results at the 110 min time point of a reaction to which Inh-2 protein was added. The Hoechst staining showed condensed chromatin and the Hoechst/phase contrast showed that the nuclear envelope was absent at this time point indicating that the reaction was in M-phase.

Overall, these results demonstrate that addition of Inh-2 protein induces an M-phase arrest in *Xenopus* cycling egg extracts, with sustained M-phase nuclear morphology, activation of the p42MAPK pathway and phosphorylation of M-phase specific phosphoproteins.

3.3 Cdc2 Kinase Activity Levels Fall and Cyclin B is Degraded Followed by an Increase in the Levels of Both in the Inh-2-Induced M-phase-Arrested Egg Extracts

In order to measure Cdc2 kinase activity, the samples taken from the reactions to which buffer and Inh-2 protein were added (Fig. 5A) were assayed using a H1K phosphorylation assay. This assay tests preparations for their ability to phosphorylate the exogenous protein histone H1 *in vitro* in the presence of radiolabelled γ-^{32}P-ATP (Lohka *et al.*, 1988).

The kinase reactions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography (Fig. 6A) and quantification by phosphorimager (Fig. 6B). In the reaction to which buffer was added, Cdc2 activity undergoes two cycles with peaks of activity at 40 and 90 min (Fig. 6A, left panel). In the reaction to which rabbit Inh-2 was added, Cdc2 activity fell at 60 min and

increased after this point (Fig. 6A, right panel). One of the requirements for Cdc2 kinase activity is its association with Cyclin proteins (reviewed in section 1.1.3). To determine if the fall in Cdc2 kinase activity (Fig. 6A, B) could be related to the level of Cyclin B protein in these samples, reactions were immunoblotted with anti-Cyclin B2 antibody (Fig. 6C). In the reaction to which buffer was added, levels of Cyclin B2 were low at 50, 100, and 110 min (Fig. 6C, left panel) coinciding with decreased Cdc2 activity (Fig. 6A, left panel, B, solid line). In the reaction to which Inh-2 protein was added, Cyclin B2 was proteolysed at 60 min (Fig. 6C, right panel) coinciding with decreased Cdc2 activity (Fig. 6A, right panel, B, broken line), indicating that the fall in Cdc2 kinase activity was due to proteolysis of Cyclin B protein. This result shows that in the reaction to which Inh-2 was added, although Cdc2 kinase activity fell and Cyclin B was degraded, the reaction continued to be arrested in M-phase. This M-phase arrest was characterized by M-phase nuclear morphology (NEBD and CC), sustained activation of the p42MAPK pathway, and sustained phosphorylation of M-phase specific phosphoproteins.

These results show that M-phase arrest induced by Inh-2 does not require sustained Cdc2 kinase activity. These results support previous reports that Cdc2 kinase inactivation does not require PP1 phosphatase activity (Felix *et al.*, 1990; Tournebize *et al.*, 1997) and that Inh-2 does not inhibit proteolysis of Cyclin B protein (Vorlaufer and Peters, 1998). In addition, they indicate that although Cdc2 kinase activity is required for phosphorylation of M-phase specific phosphoproteins it is not required for maintenance of their phosphorylation during M-phase. These results are similar to previous results in our laboratory in which the activation of the p42MAPK pathway by addition of Mos to cycling egg extracts caused an M-phase arrest despite a decrease in Cdc2 kinase activity and destruction of Cyclin B protein (Chau and Shibuya, 1998).

3.4 Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Inh-2 in *Xenopus* Cycling Egg Extracts

To determine if rabbit Inh-2-induced M-phase arrest is dependent on the concentration of Inh-2 protein, different dilutions of rabbit Inh-2 protein were added to

a *Xenopus* cycling egg extract (Fig. 7). The Cdc25 immunoblots of the results are shown in Fig. 7. Addition of Inh-2 protein at the highest concentration caused an M-phase arrest as indicated by sustained hyperphosphorylation of Cdc25 protein (Fig. 7, bottom panel). Decreasing the concentrations of Inh-2 protein (Fig. 7, fourth, third, second panels) did not cause an M-phase arrest. A likely explanation for this result is that Inh-2 protein is a known regulatory subunit of PP1 protein and binds to and inhibits PP1 phosphatase (reviewed by Cohen, 2002). It has previously been found to inhibit PP1 activity in *Xenopus* egg extracts at concentrations of 3-10 μM (Walker *et al.*, 1992; Tournebize *et al.*, 1997; Vorlaufer and Peters, 1998). Therefore, a threshold amount of Inh-2 protein is required to bind with PP1 and inhibit its activity leading to M-phase arrest.

3.5 Induction of Inh-2-Induced M-phase Arrest Requires Activation of p42MAPK

As shown in Fig. 5A (second right panel), the p42MAPK pathway became activated in the Inh-2-induced M-phase arrest. In order to test if activation of this pathway is required for this arrest, either DMSO (carrier) or PD98059 (a MEK specific inhibitor, Alessi *et al.*, 1995; Dudley *et al.*, 1995) was added to the reactions. The reactions were incubated at 21-23°C and samples were taken every 10 min for 2 hr (Fig. 8). In the reaction to which buffer and DMSO were added, the reaction underwent one cell cycle as indicated by entry into M-phase at 80 min, characterized by Cdc25 hyperphosphorylation, and M-phase nuclear morphology (NEBD and CC) (Fig. 8A, upper panel). In this reaction, p42MAPK activation was not detected by immunoblotting with anti-phospho-p44/42 antibody (Fig. 8A, lower panel). In the reaction to which Inh-2 and DMSO were added (Fig. 8B), the reaction entered M-phase at 80 min and arrested in M-phase (Fig. 8B, upper panel). p42MAPK was activated in this reaction (Fig. 8B, lower panel). Interestingly, the reaction to which Inh-2 and PD98059 were added entered M-phase at 60 min and then exited M-phase (Fig. 8C). p42MAPK was not activated in this reaction (Fig. 8C, lower panel).

This result suggests that Inh-2-induced M-phase arrest is dependent on sustained activation of the p42MAPK pathway. This result is similar to previous work

from our laboratory (Chau and Shibuya, 1999). In these experiments, addition of mutated Cdc25 (S287A) protein (this mutation does not allow the binding of 14-3-3 proteins to Cdc25) into an interphase checkpoint arrested extract overcame the interphase arrest and drove the extract into M-phase. If activation of p42MAPK was prevented by PD98059 in these extracts, the extracts failed to arrest in M-phase indicating that M-phase arrest was dependent upon p42MAPK activation.

3.6 Active p90Rsk-1 from M-phase-Arrested Unfertilized Egg Extracts Phosphorylates Rabbit Inh-2 Protein *in vitro*

p90Rsk kinase has been shown to be the kinase downstream of the p42MAPK pathway that is required for M-phase arrest in *Xenopus* cycling egg extracts (reviewed in section 1.2.2). I have shown that Inh-2-induced M-phase arrest was dependent on p42MAPK activation (Fig. 8). To determine if Inh-2-induced M-phase arrest was due to phosphorylation of Inh-2 by p90Rsk-1, I first examined if activated p90Rsk-1 can phosphorylate rabbit Inh-2 protein. p90Rsk-1 was immunoprecipitated with anti-p90Rsk-1 antibody from either an extract of interphase-arrested, activated eggs (inactive p90Rsk-1) or an extract of M-phase-arrested, unfertilized eggs (active p90Rsk-1). The p90Rsk-1 and rabbit IgG (used as negative control) immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-p90Rsk-1 antibody (Fig. 9A). The p90Rsk-1 immunoprecipitated from an extract of M-phase-arrested, unfertilized eggs was hyperphosphorylated (active) compared to p90Rsk-1 immunoprecipitated from an extract of interphase-arrested, activated eggs (inactive).

To determine the activity of the p90Rsk-1 immunoprecipitates, an *in vitro* kinase assay was done with S6 peptide as an exogenous substrate and γ-^{32}P-ATP (Fig. 9B). For quantitation of ^{32}P incorporated into substrate, the supernatants of the reactions were analyzed by scintillation counting (Fig. 9B). The results showed that the p90Rsk-1 immunoprecipitated from M-phase-arrested extract had substantially higher S6 kinase activity than p90Rsk-1 immunoprecipitated from an interphase-arrested extract and control rabbit IgG immunoprecipitates. Thus, these results showed that the hyperphosphorylated p90Rsk-1 immunoprecipitated from an extract

of M-phase- arrested, unfertilized eggs was more active than p90Rsk-1 immunoprecipitated from an extract of interphase-arrested, activated eggs.

Wang *et al.* (1995) have shown that p90Rsk-1 can phosphorylate rabbit Inh-2 *in vitro*. In addition, it has also been shown that rabbit Inh-2 protein can be phosphorylated by Cyclin B2/Cdc2 kinase (Puntoni and Moruzzi, 1995). To confirm their results, the p90Rsk-1, and Cyclin B2/Cdc2 immunoprecipitated from an extract of M-phase-arrested, unfertilized eggs and p90Rsk-1 immunoprecipitated from an extract of interphase-arrested, activated eggs were used in an *in vitro* kinase assay, with rabbit Inh-2 protein as a substrate (Fig. 10A, B). It was confirmed that the Cyclin B2/Cdc2 immunoprecipitates used possessed high activity (Fig. 22A, B). The rabbit Inh-2 protein was used since the *Xenopus* Inh-2 protein has not yet been cloned. According to quantitation of incorporated ^{32}P by phosphorimager (Fig. 10C), the rabbit Inh-2 protein phosphorylated by active p90Rsk-1 showed approximately 20-fold higher levels of ^{32}P incorporation than Inh-2 phosphorylated by p90Rsk-1 immunoprecipitated from interphase extract, and 99-fold higher than Inh-2 phosphorylated by Cyclin B2/Cdc2 kinase. These results indicated that Inh-2 is a better substrate for active p90Rsk-1 kinase than for Cyclin B/Cdc2 kinase *in vitro*.

To demonstrate that Inh-2 phosphorylation was not due to other M-phase kinases that were associated with the active p90Rsk-1 immunoprecipitates, an interphase-arrested egg extract was prepared in which p42MAPK was activated. Constitutively-active MEK protein [MEK (QP); Bottorff *et al.*, 1995; Bitangcol *et al.*, 1998] was added to an extract of interphase-arrested, activated eggs. p90Rsk-1 was immunoprecipitated from an extract of M-phase-arrested, unfertilized eggs and the MEK(QP)-activated extract of interphase-arrested extract, activated eggs. These immunoprecipitates were used in an *in vitro* kinase assay in presence of γ-^{32}P-ATP and rabbit Inh-2 protein as a substrate. The supernatants of the kinase reactions were separated on a SDS-polyacrylamide gel followed by autoradiography (Fig. 10D). The p90Rsk-1 immunoprecipitated from the MEK(QP)-activated interphase-arrested extract was also able to phosphorylate Inh-2 protein. Although, I can not exclude the existence of other kinase/kinases associated with p90Rsk-1 or CyclinB/Cdc2 immunoprecipitates that might contribute towards the overall phosphorylation

observed, our result supports a previous report that p90Rsk-1 can phosphorylate rabbit Inh-2 protein *in vitro* (Wang *et al.*, 1995).

3.7 Addition of Thiophosphorylated Inh-2 Protein Can Induce an M-phase Arrest in *Xenopus* Cycling Egg Extracts

I wished to determine if Inh-2-induced M-phase arrest (Fig. 5) could be due to phosphorylation of Inh-2 protein by p90Rsk-1, leading to an M-phase arrest. To test this hypothesis, I first determined if Inh-2 protein can be thiophosphorylated by p90Rsk-1. γ-^{35}S-ATP can be used by some kinases to phosphorylate substrates. When a substrate is phosphorylated using γ-^{35}S-ATP, the phosphorylated site is resistant to dephosphorylation because sulphur replaces the oxygen on the transferred phosphate moiety. Therefore, by irreversibly phosphorylating Inh-2 with γ-^{35}S-ATP I wished to determine if M-phase arrest induced by Inh-2 requires phosphorylation of Inh-2 by p90Rsk-1 protein.

The p90Rsk-1 immunoprecipitates from an extract of M-phase-arrested, unfertilized eggs were used in an *in vitro* kinase assay, in presence of γ-^{35}S-ATP with rabbit Inh-2 protein as a substrate (Fig. 11A, B). Kinase reactions were analyzed by SDS-PAGE followed by autoradiography. The results show that Inh-2 protein can be thiophosphorylated by p90Rsk-1.

Next, to determine if thiophosphorylated Inh-2 can induce an M-phase arrest, either buffer or thiophosphorylated Inh-2 protein from the supernatant of the kinase reaction (e.g. Fig. 11B, right panel) was added to a *Xenopus* cycling egg extract. The additions were made before incubation of the extract at 21-23°C and samples were taken for analysis every 10 min for 2 hr (Fig. 12). The samples were analyzed in the same way as in Fig. 5.

The reaction to which buffer was added, underwent one cell cycle as indicated by entry into M-phase at 60 min, characterized by Cdc25 hyperphosphorylation, M-phase nuclear morphology, and MPM-2 reactive phosphoproteins at this time point (Fig. 12A, top, bottom left panels). There is a transient low level of p42MAPK activation at 70 min when the extract was in M-phase (Fig. 12A, second left panel). The reaction to which thiophosphorylated Inh-2 protein was added entered M-phase at

70 min, and was arrested in M-phase. This M-phase arrest was characterized by sustained hyperphosphorylation of Cdc25, M-phase nuclear morphology, and sustained phosphorylation of MPM-2 reactive phosphoproteins (Fig. 12A, top, bottom right panels). The activation of the p42MAPK pathway was sustained in the thiophosphorylated Inh-2-induced M-phase arrest as shown by p42MAPK tyrosine phosphorylation (Fig. 12A, second right panel).

Fig. 12B shows cytology results of the reaction to which thiophosphorylated Inh-2 protein was added. The left pair of images (Fig. 12B, upper and lower panels) shows the cytology results at the 40 min time point. The Hoechst staining showed chromosomes starting to condense and phase contrast showed a nuclear envelope at this time point indicating that the reaction was in interphase. The right pair of images (Fig. 12B) shows the cytology results at 120 min time point. The Hoechst staining showed chromosome condensation and the phase contrast image indicated that the nuclear envelope was absent at this time point indicating that the reaction was in M-phase.

The reaction to which thiophosphorylated Inh-2 protein was added was analyzed by SDS-PAGE followed by autoradiography (Fig. 12C). Thiophosphorylated Inh-2 protein underwent post-translational modification once the reaction entered M-phase. Post-translational modification refers to changes such as phosphorylation or ubiquitination that occur to the primary structure of the protein. The modification of Inh-2 protein caused the protein to run slower on a SDS-polyacrylamide gel once the reaction entered M-phase.

The samples from a similar experiment were assayed for Cdc2 kinase activity using histone H1 as an exogenous substrate. The kinase reactions were analyzed by SDS-PAGE followed by autoradiography (Fig. 13A) and quantitation by phosphorimager (Fig. 13B). In the reaction to which buffer was added, Cdc2 activity underwent two cycles with peaks of activity at 50, and 90-100 min (Fig. 13A, left panel, 13B, solid line). In the reaction to which thiophosphorylated Inh-2 was added, Cdc2 activity decreased at 80 min (Fig. 13A, right panel, 13B, broken line), but as seen with unphosphorylated Inh-2, the reaction was arrested in M-phase, characterized

by Cdc25 hyperphosphorylation, M-phase nuclear morphology, and sustained phosphorylation of M-phase specific phosphoproteins.

To examine the level of Cyclin B protein in these reactions, the samples were monitored by immunoblotting with anti-Cyclin B2 antibody (Fig. 13C). In the reaction to which buffer was added, levels of Cyclin B2 were low at 60 and 110 min (Fig. 13C, left panel) coinciding with decreased Cdc2 activity (Fig. 13A, left panel, 13B, solid line). In the reaction to which thiophosphorylated Inh-2 protein was added, Cyclin B2 was proteolysed at 80 min (Fig. 13C, right panel) coinciding with decreased Cdc2 activity (Fig. 13A, right panel, 13B, broken line). Thus, indicating that the fall of Cdc2 kinase is due to low levels of Cyclin B protein.

These experiments do not examine the effect of only the thiophosphorylated Inh-2 in inducing this M-phase arrest. This is because the ratio of thiophosphorylated/unphosphorylated Inh-2 protein in the preparation was not determined in the kinase reactions.

Overall, these results demonstrate that addition of a preparation of Inh-2 containing thiophosphorylated Inh-2 also induced an M-phase arrest in a *Xenopus* cycling egg extract. This M-phase arrest was also characterized with M-phase nuclear morphology, sustained p42MAPK activation, sustained phosphorylation of M-phase specific phosphoproteins and did not require sustained Cdc2 activity.

3.8 Thiophosphorylated Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Thiophosphorylated Inh-2 in *Xenopus* Cycling Egg Extracts

To determine if thiophosphorylated Inh-2-induced M-phase arrest was dependent on the concentration of the thiophosphorylated Inh-2 protein, different dilutions of the thiophosphorylated Inh-2 protein were added to a *Xenopus* cycling egg extract (Fig. 14). The Cdc25 immunoblots of the results are shown in Fig. 14. Addition of the thiophosphorylated Inh-2 protein at high concentrations caused an M-phase arrest as indicated by sustained hyperphosphorylation of Cdc25 protein (Fig. 14, third, fourth, bottom panels). The lowest concentration of thiophosphorylated Inh-2 protein (Fig. 14, second panel) did not cause an M-phase arrest and the reaction entered and exited M-phase. Thus, like unphosphorylated Inh-2 protein, the

thiophosphorylated Inh-2 protein-induced M-phase arrest was dependent on the concentration of protein.

3.9 The Abilities of Inh-2 and Thiophosphorylated Inh-2 in Inducing M-phase Arrest are Similar

To compare the relative effectiveness of the thiophosphorylated and unphosphorylated Inh-2 in inducing M-phase arrest, the same *Xenopus* cycling egg extract was used to test both Inh-2 proteins at the same concentrations (Fig. 15). The Cdc25 (Fig. 15, upper panel) and activated p42MAPK (tyrosine phosphorylation) (Fig. 15, lower panel) immunoblots are shown. Addition of unphosphorylated Inh-2 and thiophosphorylated Inh-2 proteins at high concentrations (2.6, 2.1 μM) caused an M-phase arrest as indicated by sustained hyperphosphorylation of Cdc25 protein and sustained p42MAPK activation (Fig. 15B, C). The lowest concentration of these proteins (1.6 μM, Fig. 15B, C) did not cause an M-phase arrest and the reactions entered and exited M-phase. Thus, the thiophosphorylated Inh-2 protein and unphosphorylated Inh-2 protein induced M-phase arrest at similar concentrations, suggesting that there was no significant difference between the effectiveness of thiophosphorylated Inh-2 and unphosphorylated Inh-2 in inducing an M-phase arrest. These results suggest that sustained p90Rsk-1 phosphorylation of Inh-2 may not be required or sufficient for M-phase arrest.

3.10 The Rabbit and the Human Inh-2 Amino Acid Sequences Have a High Degree of Identity with the *Xenopus* Inh-2 Partial Amino Acid Sequence

PP1 regulatory proteins bind to the PP1 catalytic subunit and control its subcellular localization and activity in the cell (reviewed by Hubbard and Cohen, 1993; Cohen, 2002). The PP1 catalytic domain is highly conserved among different species. The catalytic domain of *Xenopus* γ-PP1 has 98.9 % identity with the catalytic domain of human γ-PP1. The partial nucleotide sequence of the cDNA encoding *Xenopus* Inh-2 (Genbank accession number: BI444584) was used to determine the predicted partial amino acid sequence. This sequence was then compared with rabbit and human Inh-2 full-length amino acid sequences (Fig. 16). The *Xenopus* Inh-2

partial amino acid sequence has 65.6% identity with rabbit and 63.8% identity with human Inh-2 amino acid sequences.

The p90Rsk-1 phosphorylation consensus motifs are Arg/Lys-X-Arg-X-X-Ser/Thr and Arg-Arg-X-Ser/Thr (Leighton *et al.*, 1995). The rabbit Inh-2 amino acid sequence has one potential p90Rsk-1 phosphorylation motif (a.a. 25-28). Since others and I have shown that rabbit Inh-2 protein can be phosphorylated by p90Rsk-1 *in vitro*, I examined if *Xenopus* and human amino acid sequences also contain the p90Rsk-1 phosphorylation motif. The *Xenopus* Inh-2 amino acid sequence contains one potential p90Rsk-1 phosphorylation consensus site (a.a. 24-27). However, human Inh-2 protein does not contain any p90Rsk-1 phosphorylation consensus sites (shown in balloon in Fig. 16).

3.11 Active p90Rsk-1 from M-phase-Arrested Unfertilized Egg Extracts Does Not Phosphorylate Human Inh-2 Protein *in vitro*

Since human Inh-2 protein does not contain a p90Rsk-1 phosphorylation consensus site, if p90Rsk-1 was targeting only its consensus motif, then human Inh-2 should not be phosphorylated by p90Rsk-1.

The active p90Rsk-1 immunoprecipitated from an extract of M-phase-arrested, unfertilized eggs was used in an *in vitro* kinase assay, in presence of γ-^{35}S-ATP with human and rabbit Inh-2 proteins as substrates (Fig. 17). The reactions were analyzed by SDS-PAGE followed by autoradiography (Fig. 17A,B) and quantified by phosphorimager for incorporation of ^{35}S (Fig. 17C). The level of ^{35}S incorporation into the human Inh-2 protein was extremely low when compared to rabbit Inh-2 protein. Although the human Inh-2 protein has a high degree of identity with rabbit Inh-2 protein (Fig. 16), it does not contain the p90Rsk-1 consensus site and did not become phosphorylated by p90Rsk-1 *in vitro*. Therefore, these results suggest that the only site in Inh-2 that can be phosphorylated by p90Rsk-1 is within the consensus motif. However, this result does not exclude the possibility that phosphorylation of other Ser/Thr residues present in rabbit Inh-2 (outside the consensus motif) that are not present in human Inh-2 could contribute towards some of the phosphorylation that were observed in my experiments.

3.12 Addition of Human Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extracts

Since human Inh-2 protein does not become phosphorylated by p90Rsk-1, it was next determined if it can induce an M-phase arrest in a *Xenopus* cycling egg extract. The experiments were analyzed and conducted in the same way as in Fig. 5A, except that human Inh-2 instead of rabbit Inh-2 was added to the reaction.

The reaction to which buffer was added, is the same data shown in Fig. 5A left panel. The reaction to which human Inh-2 protein was added entered M-phase at the same time as the buffer reaction, but was arrested in M-phase as characterized by sustained hyperphosphorylation of Cdc25 (Fig. 18, upper right panel). The p42MAPK pathway was activated in the human Inh-2-induced M-phase arrest as characterized by p42MAPK tyrosine phosphorylation (Fig. 18, lower right panel).

The samples were also assayed for Cdc2 activity (Fig. 19). The kinase reactions were analyzed by SDS-PAGE followed by autoradiography (Fig. 19A) and quantitation by phosphorimager (Fig. 19B). The reaction to which buffer was added is the same as that depicted in Fig. 6. In the reaction to which human Inh-2 was added, Cdc2 activity decreased after the peak at 40 min (Fig. 19A, right panel, 19B, broken line).

To examine the levels of Cyclin B protein, these samples were monitored by immunoblotting with anti-Cyclin B2 antibody (Fig. 19C). In the reaction to which human Inh-2 protein was added, Cyclin B2 was low at 50 and 60 min (Fig. 19C, right panel) correlating with decreased Cdc2 activity (Fig. 19A, right panel, 19B, broken line), indicating that the fall in Cdc2 kinase activity was due to low levels of Cyclin B protein. Thus, the reaction to which human Inh-2 was added arrested in M-phase, characterized by Cdc25 hyperphosphorylation, sustained p42MAPK activation at these time points, despite the decrease of Cdc2 activity.

Since p90Rsk-1 did not phosphorylate human Inh-2 protein *in vitro*, this result suggested that phosphorylation of the serine within the p90Rsk-1 consensus motif is not required for the Inh-2-induced M-phase arrest. In addition, these results suggested that M-phase arrest was induced by Inh-2 via activation of the p42MAPK pathway.

3.13 Human Inh-2 Antibody Can Immunoprecipitate Two Phosphorylated Proteins from *Xenopus* Egg Extract with Molecular Weights Similar to Rabbit Inh-2 Protein

To determine if anti-(human) Inh-2 antibody can recognize the endogenous *Xenopus* Inh-2 protein on a Western blot, a *Xenopus* cycling egg extract in interphase of cell cycle along with human Inh-2 protein were analyzed by SDS-PAGE and immunoblotted with anti-(human) Inh-2 antibody (Fig. 20A). Many proteins were detected after immunoblotting with the anti-(human) Inh-2 antiserum. This antibody was not able to specifically detect the endogenous *Xenopus* Inh-2 protein in *Xenopus* cycling egg extracts on a Western immunoblot. To further analyze this antibody and to determine if the antibody is able to recognize the endogenous *Xenopus* Inh-2 protein in its native form, the anti-(human) Inh-2 antibody was used in an immunoprecipitation experiment using a *Xenopus* cycling egg extract (Fig. 20B). The Cyclin B1 antibody was used as a negative control since both antibodies were raised in sheep. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-(human) Inh-2 antibody. The anti-(human) Inh-2 antibody was able to immunoprecipitate a protein band with a molecular weight similar to rabbit and human Inh-2 proteins that the anti-Cyclin B1 antibody was not able to immunoprecipitate.

Next, I used another method to determine if the antibody can recognize the *Xenopus* endogenous Inh-2 protein. Inh-2 protein is a heat-stable protein that remains soluble after boiling (Huang and Glinsmann, 1976). Since boiling denatures and precipitates the majority of other proteins, a *Xenopus* cycling egg extract was boiled, then centrifuged, and the supernatant (soluble portion) was separated from the pellet. Since both *Xenopus* and rabbit Inh-2 proteins contain the p90Rsk-1 phosphorylation consensus site (Fig. 16) and rabbit Inh-2 protein can be phosphorylated by p90Rsk-1 (Fig. 10), it was determined if *Xenopus* Inh-2 in the boiled supernatant can be phosphorylated by p90Rsk-1. The p90Rsk-1 immunoprecipitates from both an extract of interphase-arrested, activated eggs and an extract of M-phase-arrested, unfertilized eggs and Cyclin B2/Cdc2 kinase were used in an *in vitro* kinase reaction in presence of γ-^{32}P-ATP and the supernatant of boiled cycling egg extract (Fig. 21A, B).

Although, majority of proteins were precipitated by boiling, there were still many proteins left in the supernatant. There were more phosphorylated proteins in the boiled supernatant of active p90Rsk-1 compared to inactive p90Rsk-1 and Cyclin B2/Cdc2 kinase (Fig. 21B). The p90Rsk-1 phosphorylated Inh-2 protein was prepared by an *in vitro* kinase reaction in presence of γ-^{32}P-ATP with rabbit Inh-2 protein as a substrate (e.g. Fig. 10). The supernatant of this kinase reaction and p90Rsk-1 phosphorylated-supernatant of boiled egg extract were immunoprecipitated with anti-(human) Inh2 antibody. The anti-(human) Inh-2 antibody was able to immunoprecipitate two phosphorylated proteins from the active p90Rsk-1 phosphorylated-supernatant with molecular weights similar to phosphorylated rabbit Inh-2 protein immunoprecipitated with anti-(human) Inh-2 antibody (Fig. 21C, D). This result suggests that anti-(human) Inh-2 antibody can recognize two phosphorylated proteins from p90Rsk-1 phosphorylated supernatant of boiled cycling egg extract that might be the *Xenopus* Inh-2 proteins.

In summary, it is difficult to confirm that the two phosphorylated proteins on Fig. 21D are the same as the protein immunoprecipitated by anti-(human) Inh-2 antibody in Fig. 20B. There is a wider gap between the phosphorylated proteins and rabbit Inh-2 protein in Fig. 21D as compared to the faint protein band and rabbit Inh-2 protein on Fig. 20B. The proteins in both Figures 20 and 21 were separated on a 17.5% SDS-polyacrylamide gel but in Figure 20, the gel used was a mini gel and in Figure 21 the gel used was a full-sized gel. Due to these differences it is hard to conclude if the proteins on these Figures are the same proteins and if they are Inh-2 protein. On Fig. 21D, there are two phosphorylated bands with similar molecular weights to rabbit Inh-2 protein, that suggest there might be two isoforms of Inh-2 protein or other forms of the same protein present in the boiled supernatant. *Xenopus laevis* is a tetraploid (4N) species, and multiple isoforms of the same protein could exist in these species, thus the other band might be another isoform of Inh-2 protein. It could also be that *Xenopus* Inh-2 protein undergoes post-translational modification such as cleavage of the protein. Thus, further experiments need to be done to conclude if anti-(human) Inh-2 antibody can specifically detect the native *Xenopus* Inh-2 protein.

3.14 Cyclin B2/Cdc2 Kinase Immunoprecipitated from Extracts of M-phase-Arrested Unfertilized Eggs Phosphorylates Histone H1, PP1(WT) and PP1(R308A) but not PP1(T311D) *in vitro*

One of the requirements of Cdc2 kinase activation/inactivation is its binding with mitotic Cyclin proteins (reviewed by Kishimoto and Okumura, 1997). One procedure to isolate active Cdc2 kinase is to immunoprecipitate Cyclin B2 protein from an extract of M-phase-arrested, unfertilized eggs. The activity of Cdc2 kinase bound to Cyclin B2 protein was determined in an *in vitro* kinase assay in presence of γ-^{32}P-ATP with histone H1 as an exogenous substrate (Fig. 22). The kinase reactions were analyzed by SDS-PAGE followed by autoradiography (Fig. 22A) and quantitation by phosphorimager (Fig. 22B). Once the Cyclin B immunoprecipitates were confirmed to be active, they were used for other *in vitro* kinase assays (e.g. Fig. 10).

I also determined if PP1 mutated on arginine (R308A) can be phosphorylated by Cdc2 kinase. It has already been shown that Cdc2 kinase can phosphorylate the mammalian α-PP1 protein on threonine 320 *in vitro* (corresponding to threonine 311 in γ-PP1) and this phosphorylation causes an inhibition of its phosphatase activity (Dohadwala *et al.*, 1994). To confirm Cdc2 kinase phosphorylation of PP1 protein on this site, Cyclin B2 immunoprecipitates from an extract of M-phase-arrested, unfertilized eggs was used in an *in vitro* kinase assay in the presence of γ-^{32}P-ATP with human PP1 (wild type), PP1 (T311D), and PP1 (R308A) as exogenous substrates (Fig. 22C, D). The kinase reactions were analyzed by SDS-PAGE followed by autoradiography (Fig. 22C, D) and quantitation by phosphorimager (Fig. 22E). Overall, I confirmed that Cdc2 kinase can phosphorylate the human PP1 (wild type) and does not phosphorylate the human PP1 mutated on threonine 311 (T311D) *in vitro*. In addition, I also showed that the human PP1 (R308A) can be phosphorylated by Cdc2 kinase *in vitro*.

CHAPTER 4:

Chapter 4: Discussion

4.1 Inh-2-Induced an M-phase Cell Cycle Arrest in *Xenopus* Cycling Egg Extracts

Inhibitor-2 (Inh-2) is a 23 kDa protein that has been shown to inhibit the catalytic subunit of PP1, and not the other catalytic subunits of the protein phosphatase family (reviewed by Cohen, 2002). My results have shown that addition of rabbit Inh-2 protein to *Xenopus* cycling egg extracts induced an M-phase arrest of the cell cycle. The Inh-2-induced M-phase-arrested egg extracts showed sustained M-phase nuclear morphology (chromosome condensation and nuclear envelope breakdown), activation of the p42MAPK pathway, and phosphorylation of M-phase specific phosphoproteins (Fig. 5). In addition, Cyclin B protein underwent degradation that led to inactivation of the Cyclin B/Cdc2 complex in Inh-2-induced M-phase arrest. Most importantly, I showed that this M-phase arrest requires MAPK activation (Fig. 8). I next showed that although p90Rsk-1 phosphorylates rabbit Inh-2 *in vitro*, human Inh-2 is a poor substrate for p90Rsk-1. In addition, p90Rsk-thiophosphorylated Inh-2 and human Inh-2 both induced M-phase arrest in *Xenopus* cycling egg extracts. The p90Rsk-1 thiophosphorylated Inh-2 had the same effectiveness in inducing an M-phase arrest as unphosphorylated Inh-2 protein. These results suggest that p90Rsk phosphorylation of Inh-2 is not solely required to induce an M-phase arrest.

Overall, my results show induction of a MAPK-dependent M-phase arrest by a specific inhibitor of protein phosphatase-1 (PP1), Inh-2 protein, in *Xenopus* cycling egg extracts.

4.2 Requirement of PP1 Activity During the Cell Cycle and its Inhibition by Inh-2

Inh-2 protein has been shown to inhibit PP1 activity in *Xenopus* egg extracts at a concentration of 3-10 μM (Walker *et al.*, 1992; Tournebize *et al.*, 1997; Vorlaufer and Peters, 1998). Although, I did not determine PP1 activity, the concentrations of Inh-2 that were used to induce an M-phase arrest in my experiments were within the range that had been reported to inhibit PP1 activity. I have shown that Inh-2-induced

M-phase arrest was dependent on the concentration of Inh-2 protein in *Xenopus* cycling egg extracts (Fig. 7). I found that a minimum concentration of at least 2 μM of Inh-2 was required to induce an M-phase arrest. Therefore, my results show that a minimum concentration of Inh-2 was required to induce M-phase arrest, and since this concentration is similar to that previously found to inhibit PP1 activity, my results are likely due to inhibition of PP1 by Inh-2 protein.

As indicated in the introduction, PP1 activity is required for exit from M-phase. It has been shown that PP1 mutants in fission yeast (Ishii *et al.*, 1996), *Aspergillus nidulans* (Doonan and Morris, 1989) and *Drosophila* (Axton *et al.*, 1990) were able to induce M-phase arrest. My results are consistent with a requirement for PP1 activity during exit from M-phase. Although induction of an M-phase arrest by Inh-2 protein had not been shown until now, there have been reports regarding the effects of Inh-2 on the cell cycle. By inactivation of PP1, Inh-2 protein has been shown to inhibit DNA replication, overcome DNA checkpoint arrest and cause shortening of microtubules during anaphase (Walker *et al.*, 1992; Tournebize *et al.*, 1997; Vorlaufer and Peters, 1998).

In contrast to my results, Walker *et al.* (1992) have shown that the addition of rabbit Inh-2 protein to *Xenopus* egg extracts caused an early entrance into M-phase, whereas in my experiments, the extract to which Inh-2 had been added entered M-phase at the same time as the controls to which buffer had been added. Since their *Xenopus* extract was unable to undergo a complete cell cycle, they did not examine if Inh-2 was able to induce an M-phase arrest. The differences between my results and theirs may be due to the different types of extracts used in these experiments. The *Xenopus* egg extract that they used contained a high concentration of sperm nuclei and more resembled a "somatic" cell extract, with G1 and G2 phases, and the ability to respond to cell cycle checkpoints. Thus, it could be that inhibition of PP1 activity affected possible checkpoints mechanisms in G1 or G2 phases resulting in the accelerated entry into M-phase seen in their experiments, whereas these mechanisms were not activated in the "embryonic" extract used in my experiments and, therefore, not affected.

From previous reports it can be speculated that PP1 activity is not only required for exit from M-phase but it is also required during DNA replication. Since, I have not examined the status of DNA replication in my experiments, I cannot exclude the hypothesis that Inh-2-induced M-phase arrest seen in my experiments might be due to delay or defects in DNA replication caused by inactivation of PP1 activity during interphase.

For example, PP1 activity is required for dephosphorylation of inactive, hyperphosphorylated chromatin assembly factor-1 (CAF-1). The dephosphorylated and activated CAF-1 is required for nucleosome assembly during DNA replication in human cell extracts (Keller and Krude, 2000). Although CAF-1 is not required during the actual DNA synthesis, it is required during nucleosome formation that takes place during DNA replication. Inhibition of PP1 protein by Inh-2 in presence of CAF-1 caused reduction of nucleosome assembly during DNA replication. CAF-1 is a protein complex that helps to assemble nucleosomes by using Histone H3 and H4 proteins (reviewed by Adams and Kamakaka, 1999). Although, addition of okadaic acid has been shown to decrease DNA synthesis in these experiments, it needs to be further investigated if CAF-1 inactivation is responsible for a decrease in DNA synthesis (Keller and Krude, 2000). It has been shown that CAF-1 associates with replication fork-associated protein called proliferating cell nuclear antigen (PCNA) (Shibahara and Stillman, 1999). One working hypothesis is that CAF-1 associates with PCNA and is responsible for newly-formed nucleosomes during DNA replication. Although, it has not been shown if inactivation of CAF-1 would causes a decrease in DNA synthesis itself, this result supports the requirement of PP1 activity during interphase specifically for nucleosome formation during DNA replication. If CAF-1 is involved in the actual decrease in DNA synthesis, one hypothesis could be that the decrease in nucleosome assembly might act as a negative feedback to decrease DNA synthesis.

The requirement for PP1 activity during DNA replication suggests that one way for PP1 inactivation in my experiments to cause an M-phase arrest could be due to defects caused during DNA replication by PP1 inactivation that would lead to activation of the p42MAPK pathway to induce an M-phase arrest. One way to

examine this hypothesis is to first establish if addition of Inh-2 can induce an M-phase arrest in *Xenopus* cycling egg extract with high number of sperm nuclei (an extract that resembles the somatic extract and can respond to checkpoint mechanisms). If Inh-2 was able to induce an M-phase arrest in these extracts then it can be examined if addition of Inh-2 protein right before the entry of this extract into M-phase can still induce an M-phase arrest. This way DNA replication has already taken place in the extract. If inactivation of PP1 during the entry into M-phase still induced an M-phase arrest this suggests that the M-phase arrest is not likely due to defects caused by inactivation of PP1 activity during DNA replication.

It is important to examine in my experiments when PP1 becomes inactivated and if this inactivation is sustained throughout the assay. One way to examine this is to measure PP1 activity throughout the assay by taking aliquots during the incubation period from the Inh-2 added to *Xenopus* cycling egg extracts and use an exogenous substrate such as phosphorylated phosphorylase a (a substrate of PP1 protein) to measure the PP1 activity. Overall, my results suggest that induction of M-phase arrest by Inh-2 in *Xenopus* cycling egg extracts is due to PP1 inactivation, but exactly how this leads to an M-phase arrest needs to be further investigated.

4.3 Inh-2-Induced M-phase Arrest is Dependent on MAPK Activation

Interestingly, my results suggest that one way for PP1 inactivation to induce an M-phase arrest is through activation of the p42MAPK pathway (Fig. 8). In *Xenopus* cycling egg extracts, p42MAPK normally becomes transiently activated after the peak of Cyclin B/Cdc2 kinase (MPF) activation (Minshull *et al.*, 1994; Takenaka *et al.*, 1997; Bitangcol *et al.*, 1998; Guadagno and Ferrell, 1998). According to my results, in Inh-2-induced M-phase arrest, the extract entered M-phase at the same time as the control extracts (Fig. 5), but the M-phase arrest occurred after activation of the MAPK pathway, which was sustained during the arrest. In addition, the timing of p42MAPK activation was similar to the control extract and was not delayed or prematurely activated (Fig. 5). It has been reported that PP2A (another member of the protein phosphatase family) and not PP1 is the phosphatase for MAPK protein (Sohaskey and Ferrell, 1999). This suggests that PP2A instead of PP1 is a

phosphatase for MAPK and that PP1 inactivation would not directly affect p42MAPK protein activity. In my experiments I showed that inactivation of the p42MAPK pathway by PD98059 drug does not induce an M-phase arrest (Fig. 8). Since PD98059 inhibits activation of the MEK protein (the upstream kinase of p42MAPK), it can be hypothesized that the Inh-2-induced M-phase arrest is due to sustained activation of MEK protein that keeps the p42MAPK pathway activated. In support of this hypothesis it has been shown recently that inhibition of PP1 phosphatase in rat pituitary tumor cells (GH3B6) caused sustained activation of MEK protein that has led to sustained activation of the downstream MAPK pathway (Manfroid *et al.*, 2001).

Although Cdc2 kinase activity is required for phosphorylation of numerous proteins either directly, or indirectly through activation of other kinases during entry into M-phase, sustained Cdc2 activity is not required for maintaining the phosphorylated state of these proteins (Chau and Shibuya, 1998, 1999). My results also showed that Inh-2-induced M-phase arrest caused inhibition of the overall dephosphorylation of M-phase specific phosphoproteins (Fig. 5A, bottom right panel). This is consistent with results from experiments using *Aspergillus nidulans* that showed that PP1 may be required for dephosphorylation of MPM-2 antigens during mitosis (Doonan and Morris, 1989). Using immunofluorescence with MPM-2 antibody, these authors showed that mutation of bimG11 (a homologue of PP1 in *Aspergillus nidulans*) caused increased MPM-2 staining as compared to control. M-phase specific phosphoproteins in the M-phase-arrested *Xenopus* egg extracts were dephosphorylated by a phosphatase activity in interphase-arrested egg extracts (Che *et al.*, 1998). This phosphatase activity in interphase egg extracts was inactivated by okadaic acid, suggested that the sustained phosphorylation of these proteins was caused by inhibition of a phosphatase activity sensitive to okadaic acid. However, they showed that the majority of the phosphatase activity towards MPM-2 phosphoproteins was not associated with PP1, which suggests that there might be other phosphatases, with one being PP2A, that are responsible for dephosphorylation of the MPM-2 reactive proteins. This might explain why the sustained MPM-2 phosphorylation in my experiments is dependent on p42MAPK activation and not on PP1 inactivation, per se. Although, PP1 activity is required during the exit from M-

phase, the results of Che *et al.* (1998) suggest that PP1 activity is not responsible for dephosphorylation of the majority of MPM-2 reactive phosphoproteins.

The sustained MPM-2 phosphorylation has also been observed when activation of the p42MAPK pathway induced an M-phase arrest after Cyclin B/Cdc2 inactivation (Chau and Shibuya, 1998). In my experiments, the MPM-2 phosphorylation did not appear prematurely before entrance into M-phase in the Inh-2-induced M-phase-arrested extracts (Fig. 5A, bottom right panel). Since the Inh-2-induced M-phase arrest is dependent on the activation of p42MAPK in my experiments (Fig. 8), these results suggest that the overall sustained MPM-2 phosphorylation is also dependent on activation of the p42MAPK pathway and is not a general inhibition of dephosphorylation of the MPM-2 phosphoproteins. Since sustained MPM-2 phosphorylation has been observed during the p42MAPK-induced M-phase arrest (Chau and Shibuya, 1998), it suggests that the MAPK pathway might also be dependent on a phosphatase activity to sustain the MPM-2 phosphorylation during the M-phase arrest. It could be possible that the p42MAPK pathway requires PP1 inactivation to keep the extract in M-phase arrest and sustain the MPM-2 phosphorylation. One way to test this hypothesis is to examine, if activation of the p42MAPK pathway and/or PP1 inactivation is required for sustained Inh-2-induced M-phase arrest. One way to examine if sustained p42MAPK activation is required for this M-phase arrest is to inactivate the p42MAPK pathway by MAPK inhibitors [e.g. UO126 drug (Favata *et al.*, 1998)] after Inh-2 has induced an M-phase arrest in *Xenopus* cycling egg extracts. In this case, the drug, UO126, will be used instead of PD98059 since, unlike PD98059, UO126 can inhibit MEK protein that has already been activated (Favata *et al.*, 1998). If inactivation of the p42MAPK pathway did cause a release from the induced M-phase arrest, this indicates that sustained activation of this pathway is required throughout the M-phase arrest. In order to examine if PP1 inactivation is required during the sustained M-phase arrest is to add PP1 protein during the M-phase arrest induced by Inh-2. If the PP1 protein causes a release of the M-phase arrest it indicates that PP1 inactivation is also required throughout the Inh-2-induced M-phase arrest. Overall, my results show that sustained

MPM-2 phosphorylation is dependent on activation of p42MAPK and not necessarily directly due to PP1 inactivation.

4.4 Phosphorylation of Inh-2 by p90Rsk Kinase is not Solely Required for Inh-2-Induced M-phase Arrest

p90Rsk has been shown to be the key kinase downstream of the MAPK pathway that is required to induce an M-phase or CSF arrest in *Xenopus* (reviewed in section 1.2; Bhatt and Ferrell, 1999; Gross *et al.*, 1999). Since Inh-2-induced M-phase arrest is dependent on the MAPK pathway (Fig. 8) and p90Rsk has been shown to be the sole kinase downstream of this pathway to induce an M-phase arrest in *Xenopus* cycling egg extracts, I hypothesized that Inh-2 phosphorylation by p90Rsk might be required for sustaining this M-phase arrest. Consistent with previous results (Wang *et al.*, 1995), I have shown that rabbit Inh-2 becomes phosphorylated by p90Rsk-1 *in vitro* (Fig. 10). This phosphorylation did not induce any change in mobility of the protein on SDS-gels (Fig. 10; Wang *et al.*, 1995). There seems to be more than one site in Inh-2 protein for p90Rsk-1 phosphorylation *in vitro*, Ser28 (within the consensus motif as previously mentioned), and Ser35 and 202 in rabbit Inh-2 protein (Wang *et al.*, 1995).

I have also shown that human Inh-2 antibody can immunoprecipitate two phosphorylated bands with molecular weights similar to rabbit Inh-2 protein from boiled supernatants of *Xenopus* egg extracts that were phosphorylated by active p90Rsk-1 (Fig. 21). This result suggests that p90Rsk can phosphorylate endogenous Inh-2 protein *in vivo*. I was next interested to examine if p90Rsk phosphorylation of Inh-2 may have an effect on M-phase arrest. The rabbit Inh-2 protein was first phosphorylated by p90Rsk using γ-^{35}S-ATP. Proteins that are phosphorylated using γ-^{35}S-ATP are resistant to dephosphorylation. γ-^{35}S-phosphorylated Inh-2 protein was used to sustain the phosphorylation of Inh-2 protein after addition to the egg extracts so that the effects of this phosphorylation on the ability of Inh-2 in inducing M-phase arrest could be examined. The anticipated result was that if the p90Rsk phosphorylation was responsible for inducing or sustaining an M-phase arrest, then thiophosphorylated Inh-2 would have been a more effective inhibitor than

unphosphorylated Inh-2 in inducing an M-phase arrest. In this case, the thiophosphorylated Inh-2 would have induced an M-phase arrest at lower concentrations as compared to unphosphorylated Inh-2 protein. My results showed that p90Rsk-1 thiophosphorylated Inh-2 induced an M-phase arrest with similar characteristics to unphosphorylated rabbit Inh-2 protein (Fig. 12 and 15). The thiophosphorylated Inh-2-induced M-phase arrest also had sustained M-phase nuclear morphology (chromosome condensation and nuclear envelope breakdown), activation of the p42MAPK pathway, and phosphorylation of M-phase specific phosphoproteins (Fig. 12). Although my results suggest that the thiophosphorylated Inh-2 had the same ability as unphosphorylated Inh-2 in inducing M-phase arrest, one potential caveat with this result is that the ratio of thiophosphorylated/ unphosphorylated Inh-2 was not determined. Thus, the amount of thiophosphorylated Inh-2 might be too low in the reaction in order to produce any detectable effect. Another way to examine the requirement of p90Rsk-1 phosphorylation is to mutate the potential phosphorylation site/sites in rabbit Inh-2 protein. Then, if the mutated rabbit Inh-2 protein does not become phosphorylated by p90Rsk-1, it can be examined if this mutant can induce or sustain an M-phase arrest in *Xenopus* cycling egg extracts. Aside from this caveat, my results suggest that p90Rsk phosphorylation of Inh-2 does not affect the Inh-2-induced M-phase arrest.

Human Inh-2 protein shares around 92% identity with rabbit Inh-2 protein, but it does not contain any potential p90Rsk-1 phosphorylation consensus sites. In place of the arginine at position 25 in the p90Rsk consensus site of rabbit Inh-2 protein, human Inh-2 contains a methionine. In addition, it does not contain any of the other potential p90Rsk-1 phosphorylation sites in rabbit Inh-2 protein (Wang *et al.*, 1995). The *in vitro* kinase assay results showed that human Inh-2 protein became phosphorylated at a very low level, suggesting that human Inh-2 was not a potential substrate for p90Rsk-1 kinase (Fig. 17). Therefore, although human Inh-2 has a high degree of identity with rabbit Inh-2, it does not have the p90Rsk phosphorylation consensus site or the other potential serine residues predicted to be phosphorylated by p90Rsk. Thus, since it did not become significantly phosphorylated, it confirms that p90Rsk phosphorylates rabbit Inh-2 only at the predicted serine residues.

Next, I examined if human Inh-2 protein can induce M-phase arrest in *Xenopus* cycling egg extracts. I showed that human Inh-2 protein was able to induce an M-phase arrest in *Xenopus* cycling egg extract (Fig. 18). In addition, according to the parameters examined, it induced an M-phase arrest in my experiments similar to that induced by rabbit Inh-2 protein. These results strongly support that p90Rsk phosphorylation is not required for Inh-2-induced M-phase arrest. If p90Rsk phosphorylation of Inh-2 was responsible for sustaining the induced M-phase arrest, then the addition of human Inh-2 protein should have not induced an M-phase arrest.

Another way to examine if the Inh-2-induced M-phase arrest is due to PP1 inactivation is to use Inhibitor-1 protein, which is another specific inhibitor of PP1 phosphatase that shares very little homology with Inhibitor-2 protein. In order to support PP1 inactivation and its dependency on the MAPK pathway to induce M-phase arrest, it would be interesting to examine if the addition of Inh-1 to *Xenopus* cycling egg extracts can also induce an M-phase arrest that would also be dependent on the MAPK pathway. If Inh-1 protein was also able to induce an M-phase arrest similar to Inh-2 protein, it supports the hypothesis that the induced M-phase arrest is solely due to inactivation of PP1 protein and not per se due to Inh-2 protein. If the Inh-1-induced M-phase arrest was also dependent on the MAPK pathway, then it would eliminate the role of the MAPK pathway having an effect on Inh-2 protein to induce M-phase arrest.

Overall, my results suggest that Inh-2 phosphorylated by p90Rsk-1 had the same ability as unphosphorylated Inh-2 in inducing M-phase arrest in cycling egg extracts. Moreover, the characteristics of the M-phase arrests obtained were indistinguishable. The human Inh-2-induced M-phase arrest result suggest that p90Rsk phosphorylation is not solely required for Inh-2-induced M-phase arrest in *Xenopus* cycling egg extracts.

4.5 The Role of Phosphorylation of Inh-2 by Other Kinases Activated During M-phase

After addition to egg extracts and the induction of M-phase arrest, the thiophosphorylated rabbit Inh-2 underwent further post-translational modifications

when the extract entered M-phase (Fig. 12C). This can be observed by the upwards mobility shift of the thiophosphorylated Inh-2 protein on the gel (Fig. 12C). The shifted, thiophosphorylated protein appeared as several protein bands with slightly different molecular masses. Most likely, the shifted proteins are due to additional phosphorylation of Inh-2 by other kinase/kinases that are activated and sustained during M-phase arrest. Since there are many shifted bands it can be speculated that there are many phosphorylated residues. According to *in vitro* results, p90Rsk-1 phosphorylation does not induce a mobility shift of Inh-2 protein (Wang *et al.*, 1995). I also did not observe any upward mobility shift of p90Rsk-1 phosphorylated Inh-2 protein (Fig. 10). It has been shown that MAPK, GSK3, and CKII can phosphorylate Inh-2 protein *in vitro,* causing Inh-2 protein to run slower on SDS-PAGE (Hemmings *et al.*, 1982; DePaoli-Roach, 1994; Park *et al.*, 1994; Wang *et al.*, 1995). Ser 86 and Ser 120/121 of Inh-2 that are phosphorylated by CKII are not predicted potential p90Rsk phosphorylation serine residues (Wang *et al.*, 1995). GSK3 and CKII are most probably inactive during M-phase (Chen *et al.*, 1997; Reviewed by Nebreda and Ferby, 2000). CKII seems to be inactive during M-phase since CKII has been shown to inhibit activation of MAPK by binding to Mos and reduce GVBD after exposure to progesterone in *Xenopus* oocytes (Chen *et al.*, 1997). I have shown that Inh-2-induced M-phase arrest required p42MAPK activation (Fig. 8). However, phosphorylation of threonine 72 of Inh-2 protein by MAPK which induces a mobility shift on SDS-PAGE has been shown to activate the Inh-2/PP1 complex *in vitro* (Wang *et al.*, 1995). Therefore, the activation of Inh-2/PP1 due to phosphorylation by MAPK is inconsistent with the presumed Inh-2/PP1 inactivation during M-phase arrest in my experiments.

It has already been shown that phosphorylation of Inh-2 by p90Rsk does not dissociate the Inh-2/PP1 complex (Wang *et al.*, 1995). One hypothesis could be that p90Rsk phosphorylation of Inh-2 is responsible for translocation of the complex in the cell. For instance mutation of sites in Inh-2 phosphorylated by GSK3/MAPK and CK II (Thr 72, Ser 86 or Ser 120/121) prevented accumulation of Inh-2 in the nucleus (Kakinoki *et al.*, 1997). It can be hypothesized that this might be one way for translocating PP1 to nucleus and their association with chromosomes. It needs to be

further investigated if the M-phase arrests in our experiments are dependent on these phosphorylations. It has also been shown that phosphorylation of another regulatory subunit of PP1, Gm (Glycogen targeting subunit) by insulin-stimulated protein kinase (ISPK) which is homologous to p90Rsk-2 cause's increase in glycogen synthase phosphatase activity by associating the Gm/PP1 complex to glycogen particles (Dent *et al.*, 1990; Lavoinne *et al.*, 1991; Sutherland *et al.*, 1993; reviewed by DePaoli-Roach *et al.*, 1994). Thus, p90Rsk phosphorylation could have other effects on Inh-2 protein that I did not examine in my assays.

Puntoni and Moruzzi (1995) showed that Cyclin B/Cdc2 kinase was able to phosphorylate purified rabbit skeletal muscle Inh-2 protein but with low stoichiometry (0.1 mol/mol) and recombinant Inh-2 protein with higher stoichiometry (1.1-1.2 mol/mol). I have shown that recombinant rabbit Inh-2 protein was phosphorylated to a very low level by active Cdc2 kinase as compared to phosphorylation by p90Rsk-1 *in vitro* (Fig. 10). Thus, my results suggest that Inh-2 protein is not a target for Cdc2 kinase in M-phase-arrested egg extracts.

Overall, further experiments or assays are required in order to determine the function of Inh-2 phosphorylation by p90Rsk and other kinases that are activated during M-phase in *Xenopus* cycling egg extracts.

4.6 Phosphorylation of PP1 Phosphatase by Cyclin B/Cdc2 during M-phase

I have confirmed that the PP1 catalytic subunit can become phosphorylated by Cyclin B/Cdc2 kinase on Thr 311 (Fig. 19). Active Cdc2 kinase has been shown to phosphorylate the mammalian PP1 catalytic subunit and this phosphorylation caused inhibition of PP1 phosphatase activity (Dohadwala *et al.*, 1994). This site can also become phosphorylated by Cdc2 kinase in an isoform of PP1 called dis2 in fission yeast *S. pombe* (Yamano *et al.*, 1994). I have also examined another mutant of PP1, in which Arg 308 was mutated to alanine. It is hypothesized that this arginine might be required for inactivation of Cdc2-phosphorylated-PP1 phosphatase activity, by changing the conformation of the protein.

The Cdc2 kinase phosphorylation of the PP1 catalytic subunit might also be required during the cell cycle. In fission yeasts, mutant dis2, which cannot be

phosphorylated by Cdc2 kinase, were not able to grow and form a colony (Yamano *et al.*, 1994). Cdc2 phosphorylation of PP1 needs to be further investigated in *Xenopus*, since in my experiments Cdc2 kinase becomes inactivated in Inh-2-induced M-phase-arrested extracts (Fig. 6). This result suggests that sustained Cdc2 phosphorylation of PP1 that leads to PP1 inactivation is not required for sustained M-phase arrest.

It seems that activity of Inh-2/PP1 can be dependent on phosphorylation of both proteins but exactly how phosphorylation regulates these activities during cell cycle needs to be further investigated. My results suggest that Inh-2-induced M-phase arrest is independent from sustained Cdc2 activity. Since sustained Cdc2 phosphorylation of PP1 might not be dependent on sustained Cdc2 activation, it needs to be further investigated if PP1 phosphorylation by Cdc2 is sustained and/or required in the Inh-2-induced M-phase arrests.

4.7 Cdc2 Kinase Inactivation and Cyclin B Degradation are not Inhibited in Inh-2-Induced M-phase Arrest

I have shown that Inh-2-induced M-phase arrest did not require sustained activation of Cdc2 kinase, since Cyclin B protein was degraded (Fig. 6). Thus, Inh-2-induced M-phase arrest does not inhibit the proteolytic machinery that leads to degradation of Cyclin B protein and inactivation of Cdc2 kinase. My results are in agreement with previous work that showed addition of rabbit Inh-2 protein did not inhibit Cdc2 kinase activity (Felix *et al.*, 1990, Tournebize *et al.*, 1997) as well as Cyclin B destruction (Vorlaufer and Peters, 1998) in *Xenopus* egg extracts. Thus, my results also suggest that Cyclin B/Cdc2 kinase activation and inactivation are independent of PP1 phosphatase activity.

One reason for inactivation of Cdc2 kinase activity in Inh-2-induced M-phase arrest might be the timing of p42MAPK activation in my extracts. In my experiments, p42MAPK became activated at the precise time when Cdc2 kinase activity levels fell and Cyclin B protein was degraded (Fig. 6). It has been found previously that if p42MAPK was activated after the peak of Cyclin B/Cdc2 kinase activity, Cdc2 kinase was inactivated and Cyclin B protein was proteolyzed but *Xenopus* cycling egg extracts remained in M-phase (Chau and Shibuya, 1998; Guadagno and Ferell, 1998).

On the other hand if p42MAPK was activated before the peak of Cyclin B/Cdc2 kinase it resulted in sustained activation of Cyclin B/Cdc2 activity (Minshull *et al.*, 1994; Abrieu *et al.*, 1996; Walter *et al.*, 1997; Chau and Shibuya, 1998, 1999). These results support the importance in timing of p42MAPK activation in relation to Cyclin B/Cdc2 kinase activity in *Xenopus* cycling egg extracts. Overall, my results suggest that proteolysis of Cyclin B protein is independent of PP1 and PP1 inactivation does not affect activity of Cyclin B/Cdc2 kinase. It also supports the previous results that inactivation of Cyclin B/Cdc2 kinase is not solely required for exit from M-phase, but inactivation of p42MAPK is required for exit from M-phase.

4.8 Summary

As mentioned in the introduction, it has already been shown that PP1 activity is required for exit from M-phase. This suggests that PP1 activity is required for dephosphorylation of kinase/s or protein/s that is necessary for exit from M-phase. Further, activation of the p42MAPK pathway has also been shown to induce an M-phase arrest. My results showed that Inh-2-induced M-phase arrest required activation of the p42MAPK pathway for Inh-2-induced M-phase arrest, suggesting the dependency of PP1 inactivation on p42MAPK activation. It would be ideal to examine next if this induced M-phase arrest requires PP1 inactivation to sustain this M-phase arrest. If PP1 inactivation is required it suggests that the p42MAPK pathway is also dependent on PP1 inactivation to induce an M-phase arrest. It can be hypothesized that the p42MAPK pathway also requires sustained PP1 inactivation to induce an M-phase arrest. Interestingly, inactivation of PP1 or activation of p42MAPK separately has been shown to shorten the length of microtubules in *Xenopus* (Tournebize *et al.*, 1997; Guadagno and Ferrell, 1998). These findings suggest that they both induce the same effects during M-phase. It also suggests that these two pathways might share common downstream pathway to induce these effects. At molecular level, exactly how PP1 and p42MAPK induce these affects still remains to be determined. My results showed that Inh-2 phosphorylation by p90Rsk-1 does not seem to be solely required for induction or sustaining of the Inh-2-induced M-phase arrest.

In summary, the major novel result found in this study is the induction of M-phase arrest by a PP1 regulatory subunit, Inhibitor-2 protein, in *Xenopus* cycling egg extracts and its dependency on the MAPK pathway. This result supports the requirement for MAPK inactivation during the exit from M-phase. Further, I showed that Inh-2 phosphorylation by p90Rsk-1 is not solely responsible for this M-phase arrest.

CHAPTER 5:

Figure 1: Schematic Diagram of *Xenopus* Oocyte Maturation

The schematic diagram represents the *Xenopus* oocyte maturation process. After exposure to progesterone, immature oocytes undergo oocyte maturation (a process involving meiotic division). This process is not completed and cells arrest during metaphase II of the second meiotic division. Fertilization releases the metaphase II arrested eggs allowing the zygotes (fertilized eggs) to undergo mitotic division.

Figure 1: Schematic Diagram of *Xenopus* Oocyte Maturation

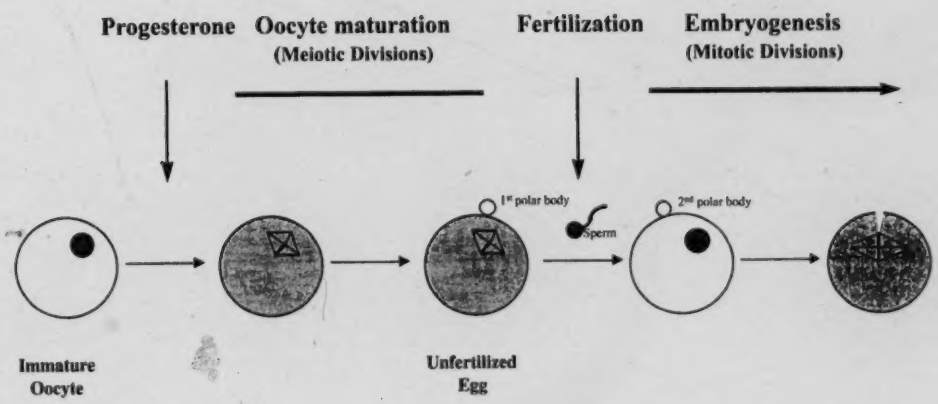


Figure 2: Schematic Diagram of MPF (Cyclin B/Cdc2) Activation and Inactivation During the *Xenopus* Early Embryonic Cell Cycle

The schematic diagram represents the process of MPF (Cyclin B/Cdc2) activation and inactivation during early embryogenesis. Cdc25 protein dephosphorylates Cdc2 kinase by removing the phosphate groups on threonine 15 and tyrosine 14. This activates MPF, driving the cell cycle into M-phase. The activated Cyclin B/Cdc2 phosphorylates substrates leading to nuclear envelope breakdown and chromosome condensation. Towards the end of M-phase, Cyclin protein is degraded leading to dissociation from Cdc2 and inactivation of MPF (Cyclin B/Cdc2).

Figure 2: Schematic Diagram of MPF (Cyclin B/Cdc2) Activation and Inactivation During the *Xenopus* Early Embryonic Cell Cycle

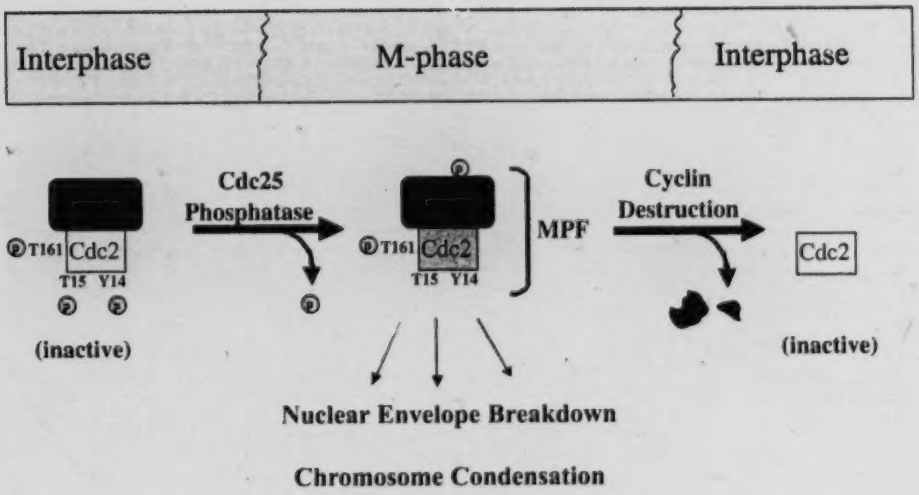


Figure 3: Schematic of *Xenopus* Cycling Egg Extract Procedure

The procedure of making a *Xenopus* cycling egg extract is shown in this schematic diagram. The M-phase-arrested, unfertilized eggs are collected, dejellied and released from M-phase arrest by electric shock. Then, the eggs are packed and crushed by centrifugation. The turbid supernatant is recentrifuged, then the cleared extract is placed in a tube along with an ATP-regenerating system and demembranated sperm nuclei.

Figure 3: Schematic of *Xenopus* Cycling Egg Extract Procedure

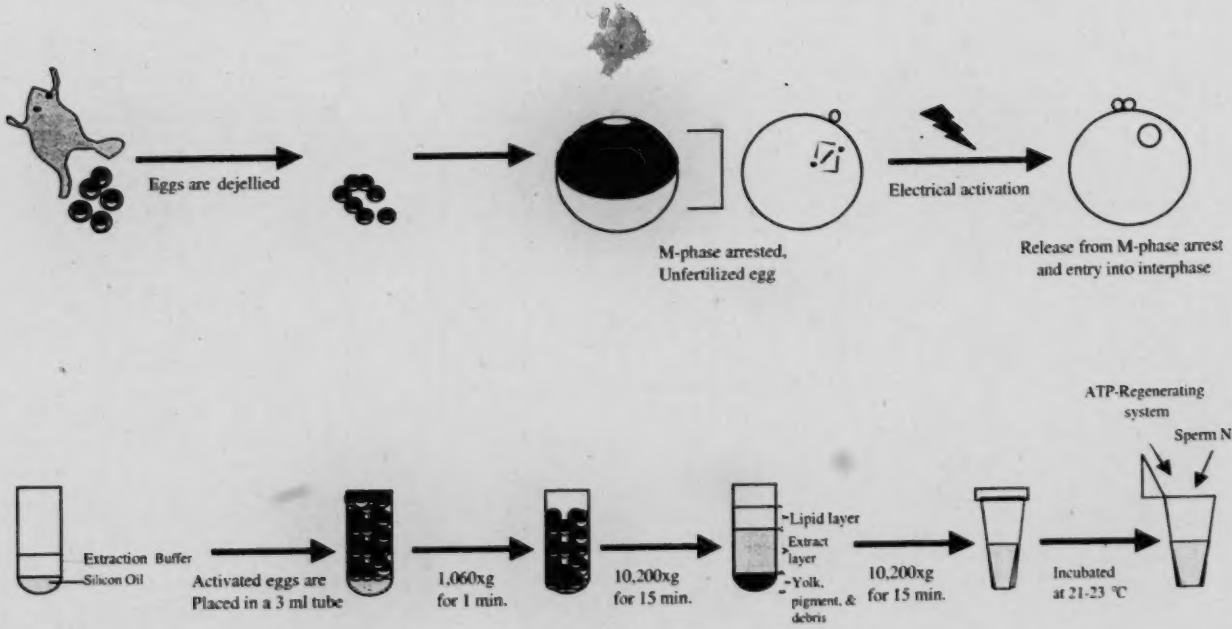


Figure 4: Purification of His-tagged Rabbit Inh-2 protein from *Escherichia coli (E. coli)*

A recombinant His-tagged rabbit Inh-2 protein was expressed and purified from *Escherichia coli* (*E. coli*). The preinduction (Pre) and post induction (Post) samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried.

A) The supernatants before (Pre-Binding) and after (Post-Binding) bead binding and the eluted samples were analyzed (as in Fig. 4A). Numbers 1 and 2 on the gel denote the first and the second eluate for each imidazole concentration.

B) The eluted fractions were analyzed by SDS-PAGE to determine which fractions contained Inh-2 protein.

C) The eluted fractions (from Fig. 4B) that contained His-tagged Inh-2 protein were pooled and dialyzed at 4°C. The protein concentration was determined at the end; samples from the concentrated His-tagged protein and different amounts of bovine serum albumin (BSA) were analyzed (as in Fig. 4A).

D) The graph representing the quantitation of BSA (from Fig. 4C) is shown. The individual values were plotted and a linear graph (least squares regression) was generated that is shown on the graph. Then, the equation of the linear graph was used to calculate the concentration of His-tagged rabbit Inh-2 protein. The average concentration from individual values was calculated. The concentration of rabbit Inh-2 protein was approximately 1.3 µg/µl for this preparation.

Figure 4: Purification of His-tagged Rabbit Inh-2 protein from *Escherichia coli* (*E. coli*)

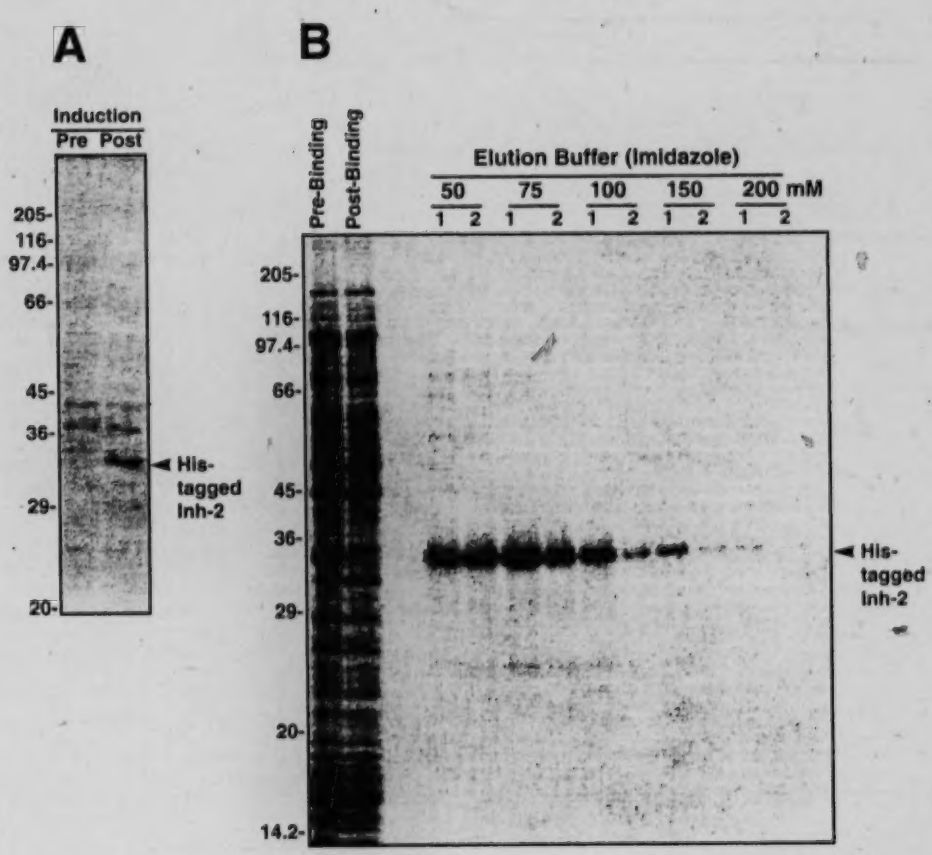
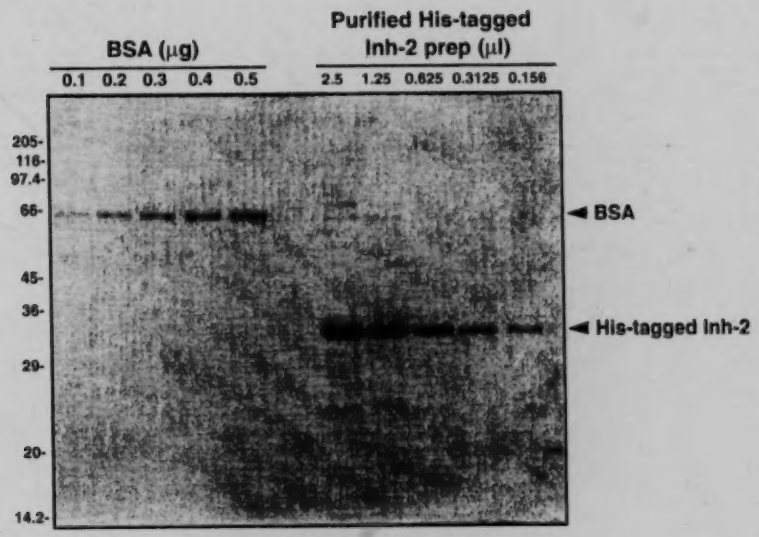
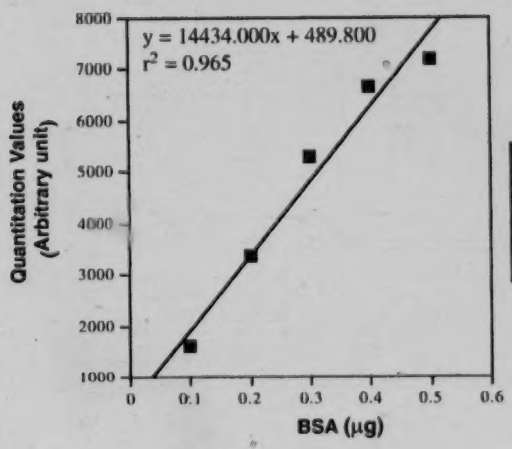


Figure 4:

C



D



μl loaded	Value	[Inh-2] μg/μl
0.3125	5800	1.17
0.156	3854	1.49

Figure 5: Addition of Rabbit Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extract

A) A *Xenopus* cycling egg extract was prepared and either buffer or rabbit Inh-2 protein was added to 14% (v/v) of the reaction. The reactions were incubated at 21-23°C and samples were taken every 10 min for analysis. Samples were analyzed by SDS-PAGE followed by immunoblotting. The top panel shows a Cdc25 immunoblot; second panel, p42MAPK tyrosine phosphorylation (4G10) immunoblot; third panel, p90Rsk-1 immunoblot; and bottom panel an MPM-2 immunoblot. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M). The p-Cdc25 arrow and pp90Rsk-1 solid line denote the hyperphosphorylated form of these proteins. The bars below the Cdc25 immunoblot indicate samples in which M-phase morphology of nuclei was observed, [nuclear envelope breakdown (NEBD) and chromosome condensation (CC)].

B) The cytology results of the reactions to which buffer and rabbit Inh-2 protein were added (from two different experiments) are shown. The Hoechst stains chromatin; Phase contrast and combined Hoechst /Phase contrast show the presence/absence of a nuclear envelope. Bar, 10 μM.

Figure 5: Addition of Rabbit Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extract

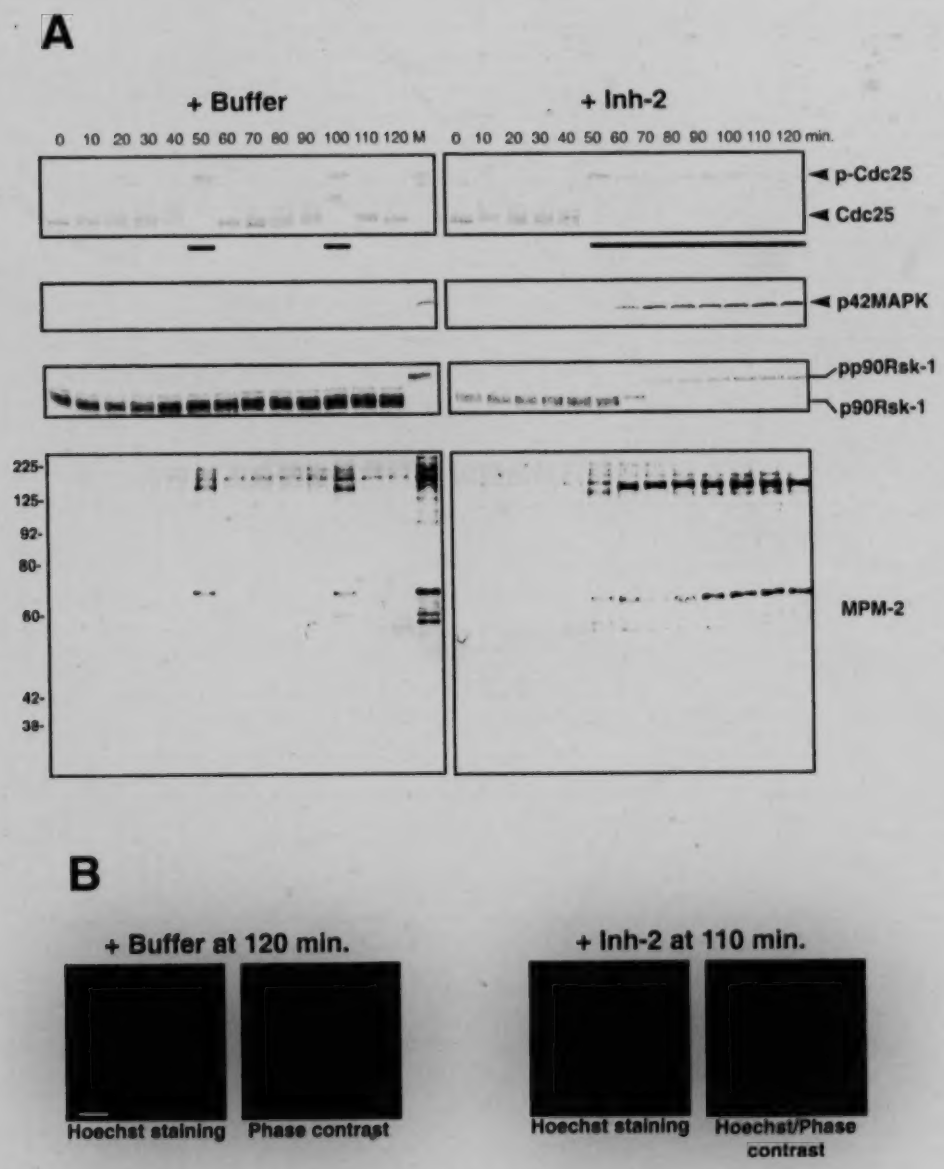


Figure 6: Cdc2 Kinase Activity Levels Fall and Cyclin B is Degraded Followed by an Increase in the Levels of Both in the Inh-2 Induced M-phase Arrested Egg Extracts

A) The samples taken from the experiment shown in Fig. 5A), were assayed for Cdc2 kinase activity. The histone H1 kinase assay was done in presence of γ-^{32}P-ATP and histone H1 as an exogenous substrate. Kinase reactions were analyzed by SDS-PAGE followed by autoradiography. The bars below the Cdc25 immunoblot indicates samples in which M-phase morphology of nuclei was observed, [nuclear envelope breakdown (NEBD) and chromosome condensation (CC)].

B) The graph represents the quantitation of ^{32}P incorporated into phosphorylated histone H1 by phosphorimager.

C) The samples from the experiment shown in Fig. 6A) were immunoblotted with anti-Cyclin B2 antibody. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M).

Figure 6: Cdc2 Kinase Activity Levels Fall and Cyclin B is Degraded Followed by an Increase in the Levels of Both in the Inh-2 Induced M-phase Arrested Egg Extracts

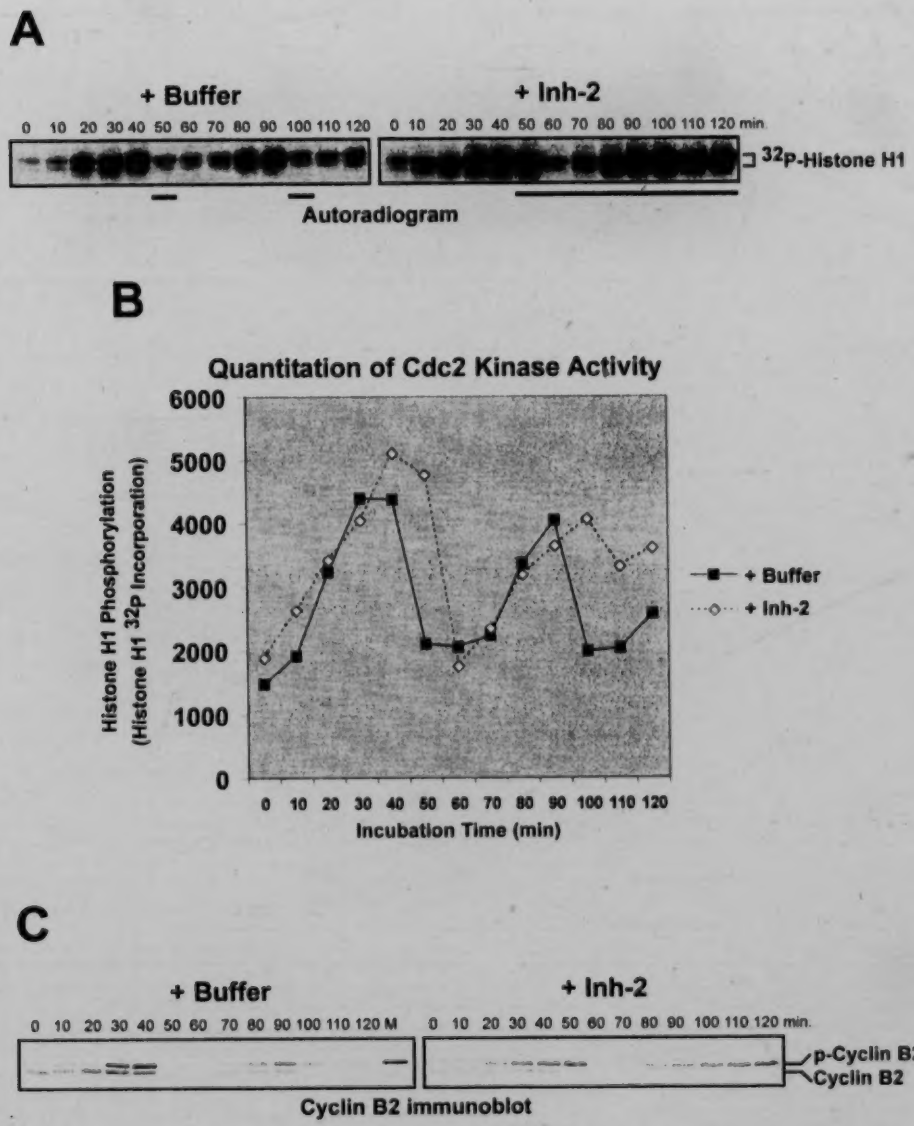
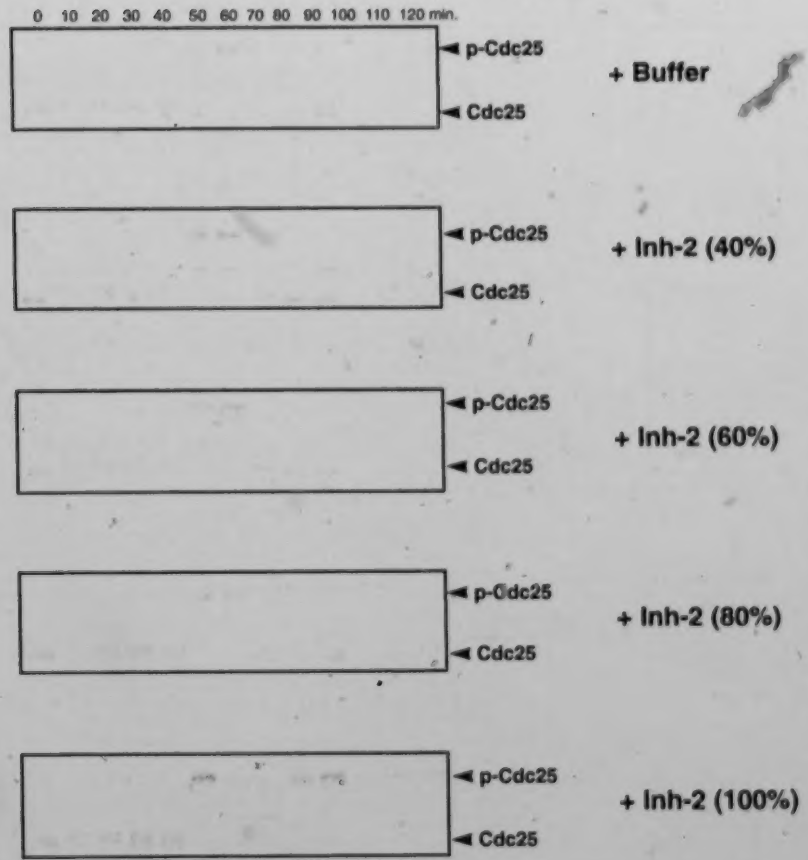


Figure 7: Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Inh-2 in the *Xenopus* Cycling Egg Extract

A *Xenopus* cycling egg extract was prepared, and either buffer or different concentrations of His-tagged rabbit Inh-2 protein was added to 5% (v/v) of the final reaction (Note: extract and reactions were prepared by E. Shibuya). Samples were analyzed by SDS-PAGE followed by immunoblotting with Cdc25 antibody. The p-Cdc25 arrow denotes the hyperphosphorylated form of this protein.

Figure 7: Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Inh-2 in the *Xenopus* Cycling Egg Extract



Cdc25 immunoblot

Figure 8: Induction of Inh-2 M-phase Arrest Requires Activation of p42MAPK

A *Xenopus* cycling egg extract was prepared and either buffer or His-tagged rabbit Inh-2 protein was added to 5% (v/v) of the final reaction. In addition, either DMSO or 300 μM (final concentration) of PD98059 was added at the same volume to these reactions. Samples were analyzed by SDS-PAGE followed by immunoblotting. The upper panel of each pair shows the Cdc25 immunoblot (p-Cdc25 arrow denotes the hyperphosphorylated form), and the lower panel shows activated p42MAPK (phospho-p44/42) immunoblot. The bars below the Cdc25 immunoblots represent samples in which M-phase morphology of nuclei was observed, [nuclear envelope breakdown (NEBD) and chromosome condensation (CC)]. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M).

A) The results of the reaction to which Buffer and DMSO were added are shown.
B) The results of the reaction to which Inh-2 and DMSO were added are shown.
C) The results of the reaction to which Inh-2 and 300 μM (final concentration) of PD98059 were added are shown.

Figure 8: Induction of Inh-2 M-phase Arrest Requires Activation of p42MAPK

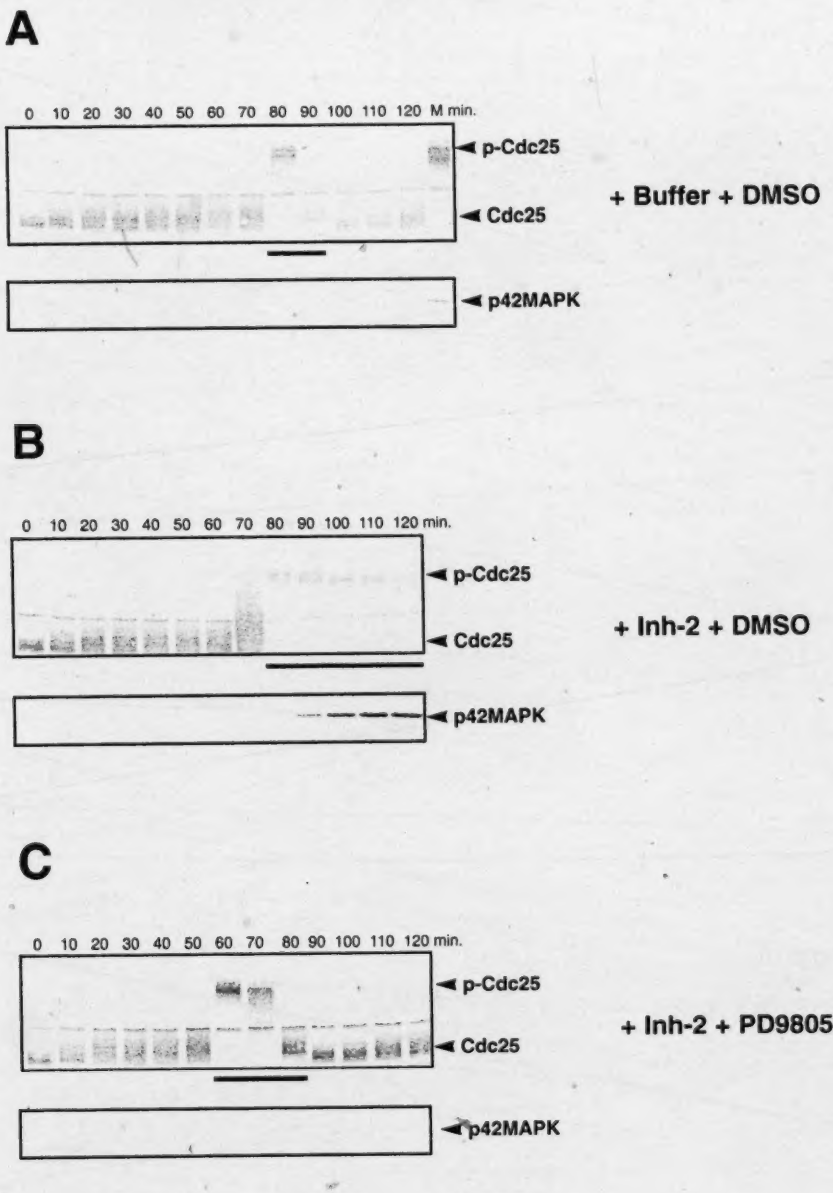
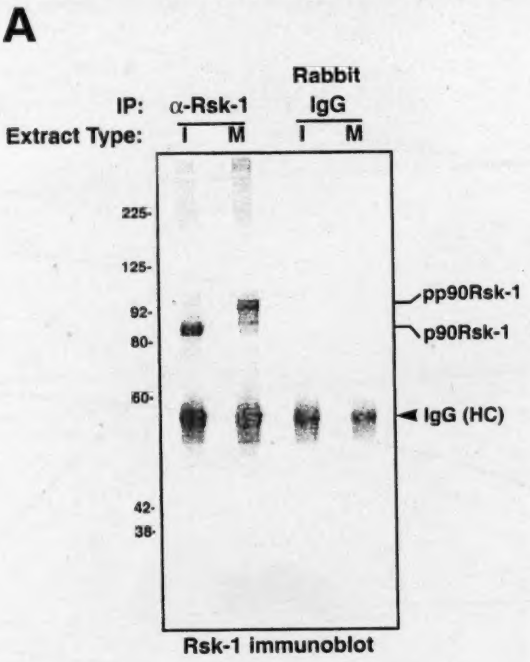


Figure 9: p90Rsk-1 is Hyperphosphorylated and Active in M-phase Arrested Unfertilized Egg Extracts

A) An extract of interphase-arrested, activated eggs (I) and an extract of M-phase-arrested, unfertilized eggs (M) were subjected to immunoprecipitation (IP) using anti-Rsk-1 antibody or rabbit IgG. Samples were analyzed by SDS-PAGE followed by immunoblotting with anti-Rsk-1 antibody. The pp90Rsk-1 black solid line denotes the hyperphosphorylated form of p90Rsk-1 and the IgG (HC) arrow denotes the heavy chain of the IgG.

B) The anti-Rsk-1 and rabbit IgG immunoprecipitates (IPs) from an extract of interphase-arrested, activated eggs (I) or an extract of M-phase-arrested, unfertilized eggs (M), of another experiment were used in an *in vitro* kinase assay. The kinase reactions were done in presence of γ-^{32}P-ATP and S6 peptide as a substrate. The graph represents the quantitation of ^{32}P incorporation using a scintillation counter.

Figure 9: p90Rsk-1 is Hyperphosphorylated and Active in M-phase Arrested Unfertilized Egg Extracts



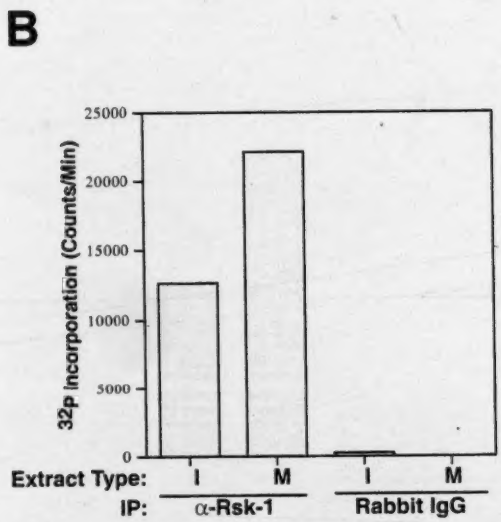


Figure 10: Active pp90Rsk-1 from M-phase Arrested Unfertilized Egg Extracts Phosphorylates Rabbit Inh-2 Protein *in vitro*

A) The p90Rsk-1 protein was immunoprecipitated (IP) using anti-Rsk-1 antibody from an extract of interphase-arrested, activated eggs (I) or an extract of M-phase-arrested, unfertilized eggs (M). The Cyclin B2 protein was immunoprecipitated (IP) using anti-Cyclin B2 antibody from an extract of M-phase-arrested, unfertilized eggs (M). These immunoprecipitates were then used in an *in vitro* kinase assay. The kinase reactions were done in presence of γ-^{32}P-ATP and rabbit Inh-2 as a substrate. The reactions were incubated for 1 hr at 22°C, then analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried. The IgG (HC) black solid line denotes the sheep antibody heavy chain IgG and the IgG (HC) black dashed line denotes the rabbit antibody heavy chain IgG.

B) The autoradiogram of the gel in Fig. 10A) is shown.

C) The graph represents the quantitation of ^{32}P incorporation into phosphorylated Inh-2 protein by phosphorimager.

D) A constitutively-active recombinant MEK [MEK(QP)] protein was added to an interphase-arrested egg extract to activate the p42MAPK pathway. Then, p90Rsk-1 was immunoprecipitated from both an extract of M-phase-arrested, unfertilized eggs (M) and Interphase + MEK(QP) egg extract. These immunoprecipitates were then used in an *in vitro* kinase assay. The kinase reactions were done in presence of γ-^{32}P-ATP and rabbit Inh-2 as a substrate. The reactions were incubated for 2 hr at 23-24°C. At the end of incubation, the reactions were centrifuged at 10,200xg for 10 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge and the supernatant (soluble portion) was removed from the pellet (beads). The samples were analyzed by SDS-PAGE, stained with Coomassie blue, destained and then dried. The autoradiogram of the gel is shown.

Figure 10: Active pp90Rsk-1 from M-phase Arrested Unfertilized Egg Extracts Phosphorylates Rabbit Inh-2 Protein *in vitro*

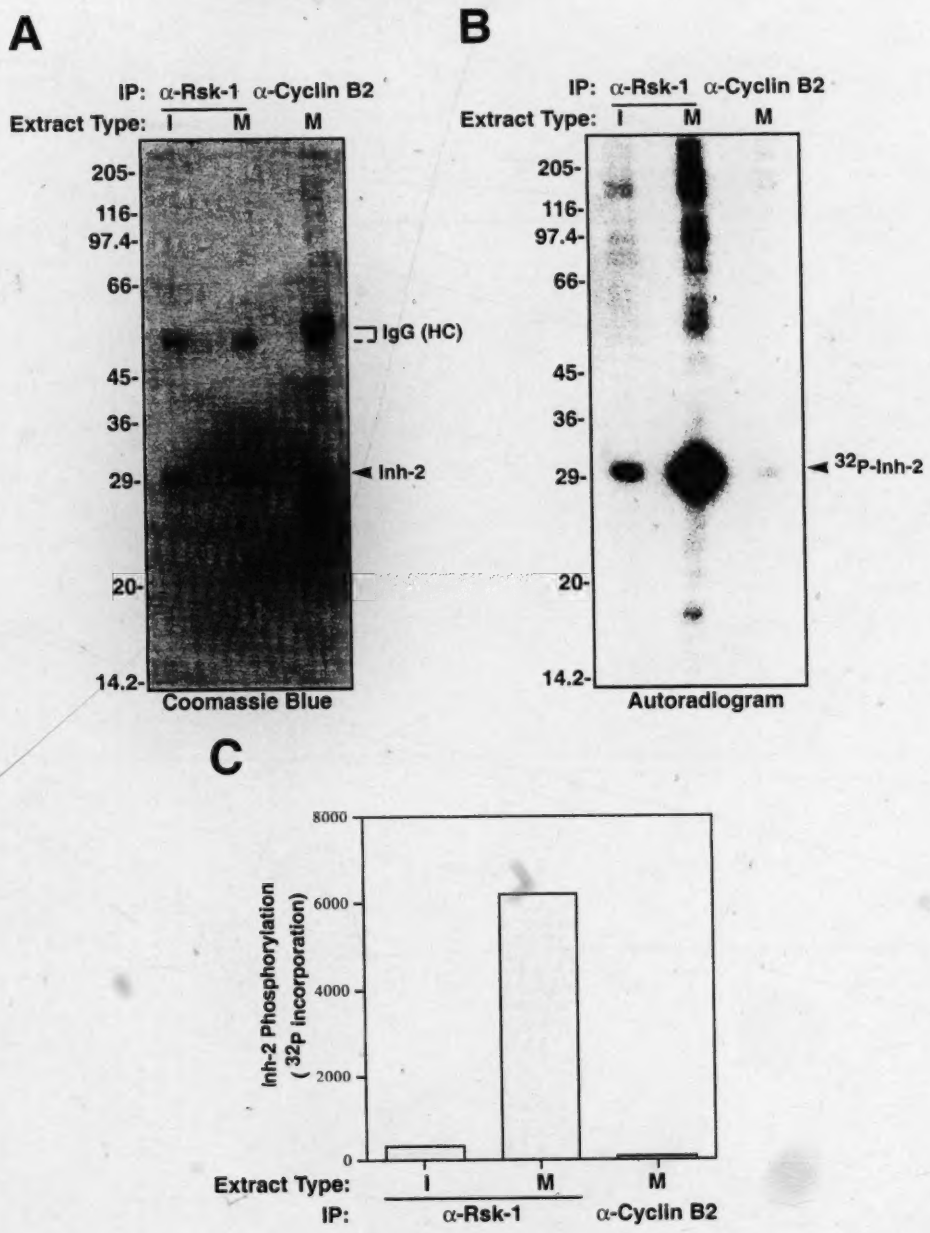


Figure 10:

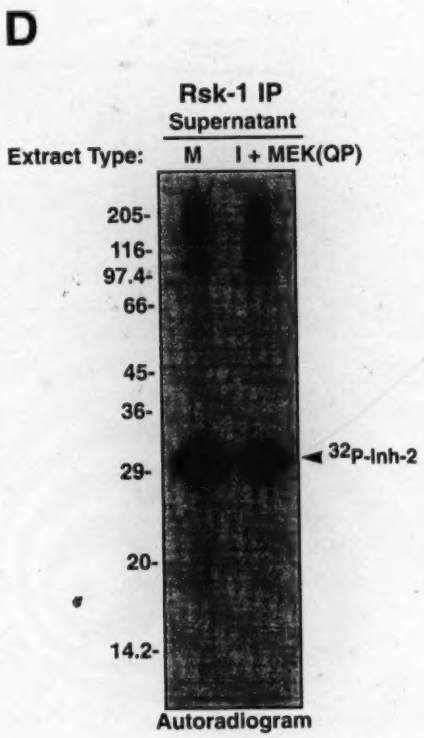

D
Rsk-1 IP
Supernatant
Extract Type:
M
I + MEK(QP)
205-
116-
97.4-
66-
45-
36-
29-
20-
14.2-
32P-Inh-2
Autoradiogram

Figure 11: Rabbit Inh-2 Protein Can Be Thiophosphorylated by Active pp90Rsk-1 *in vitro*

A) The p90Rsk-1 protein was immunoprecipitated (IP) using anti-Rsk-1 antibody from an extract of M-phase-arrested, unfertilized eggs (M). These immunoprecipitates were then used in an *in vitro* kinase assay. The kinase reaction was done in presence of γ-^{35}S-ATP and rabbit Inh-2 as a substrate. The reaction was incubated for 8 hr at 25°C. Then, a sample from a reaction mix (pellet and supernatant) was removed and the remaining reaction was centrifuged. The supernatant (soluble portion) was removed from the pellet (beads). The samples from reaction mix and supernatant were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried. Note: the Inh-2 in the supernatant sample on the gel was not visible by Coomassie staining (data not shown).

B) The autoradiograms of the gel of the reaction mix (pellet and supernatant) and supernatant in Fig. 11A) are shown.

Figure 11: Rabbit Inh-2 Protein Can Be Thiophosphorylated by Active pp90Rsk-1 *in vitro*

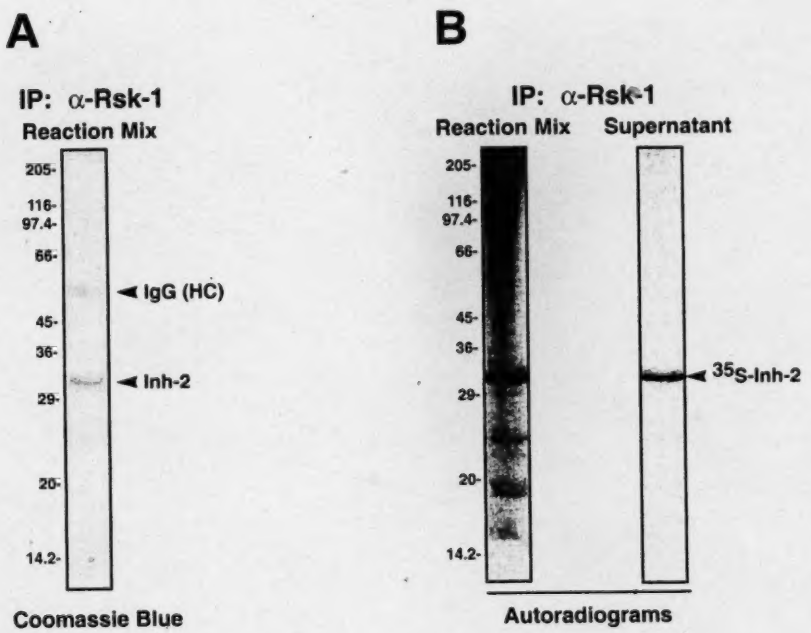


Figure 12: Addition of Thio-p-Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extract

A) A *Xenopus* cycling egg extract was prepared and either buffer or thiophosphorylated (thio-p-) rabbit Inh-2 protein was added to 14% (v/v) of the final reaction. Samples were analyzed by SDS-PAGE followed by immunoblotting. The top panel shows a Cdc25 immunoblot; second panel, p42MAPK tyrosine phosphorylation (4G10) immunoblot, and bottom panel an MPM-2 immunoblot. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M). The p-Cdc25 arrow denotes to the hyperphosphorylated form of this protein. The bars below the Cdc25 immunoblot indicate samples in which M-phase morphology of nuclei was observed, [nuclear envelope breakdown (NEBD) and chromosome condensation (CC)].

B) The cytology results of the thiophosphorylated Inh-2 protein addition to *Xenopus* cycling egg extract are shown. The Hoechst stains chromatin; Phase contrast shows the presence/absence of a nuclear envelope. Bar, 10 μM.

C) Samples from thiophosphorylated Inh-2 reaction were separated on a 17.5% SDS-polyacrylamide gel, stained with Coomassie blue, destained and then dried. The autoradiogram of the gel is shown.

Figure 12: Addition of Thio-p-Inh-2 Protein Can Induce an M-phase Arrest in *Xenopus* Cycling Egg Extract

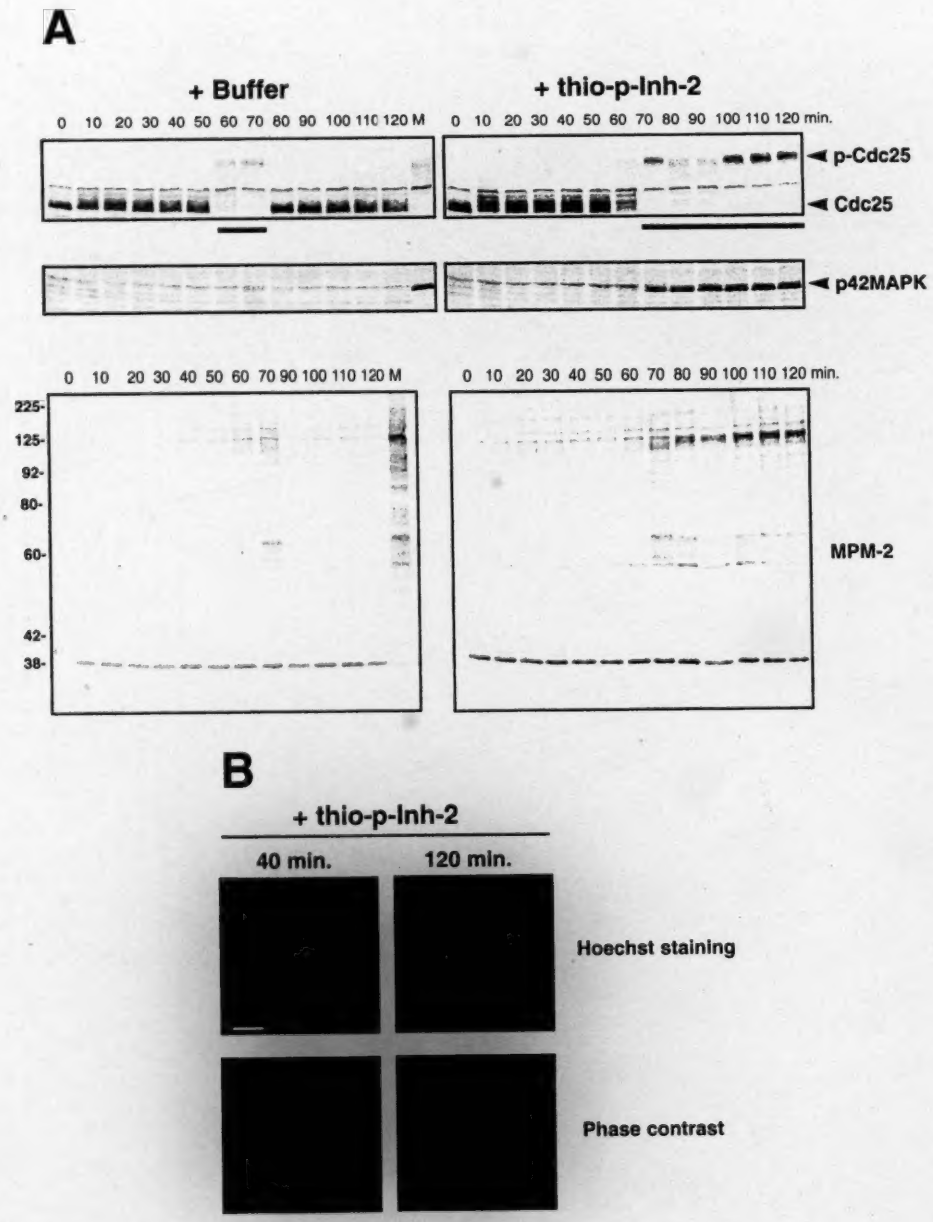


Figure 12:

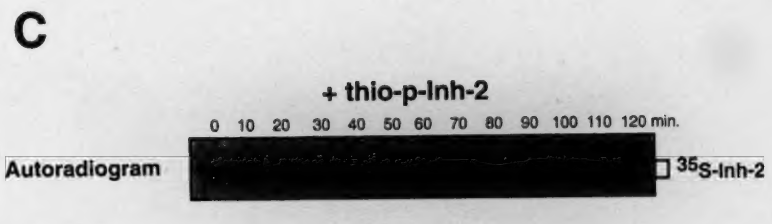

C
+ thio-p-Inh-2
0 10 20 30 40 50 60 70 80 90 100 110 120 min.
Autoradiogram
35S-Inh-2

Figure 13: Cdc2 Kinase Activity Levels Fall, and Cyclin B is Degraded Followed by an Increase in Levels of both in the Thio-p-Inh-2 Induced M-phase Arrested Egg Extracts

A) Samples from another experiment similar to Fig. 12 were assayed for Cdc2 kinase activity. The histone H1 kinase assay was done in presence of γ-^{32}P-ATP and histone H1 as an exogenous substrate. Kinase reactions were analyzed by SDS-PAGE followed by autoradiography. Note: the 70 min thiophosphorylated Inh-2 sample is missing in Fig. 13A) and B).

B) The graph represents the quantitation of ^{32}P incorporated into phosphorylated histone H1 by phosphorimager.

C) The samples from the experiment shown in Fig. 13A) were immunoblotted with anti-Cyclin B2 antibody. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M).

Figure 13: Cdc2 Kinase Activity Levels Fall, and Cyclin B is Degraded Followed by an Increase in Levels of both in the Thio-p-Inh-2 Induced M- phase Arrested Egg Extracts

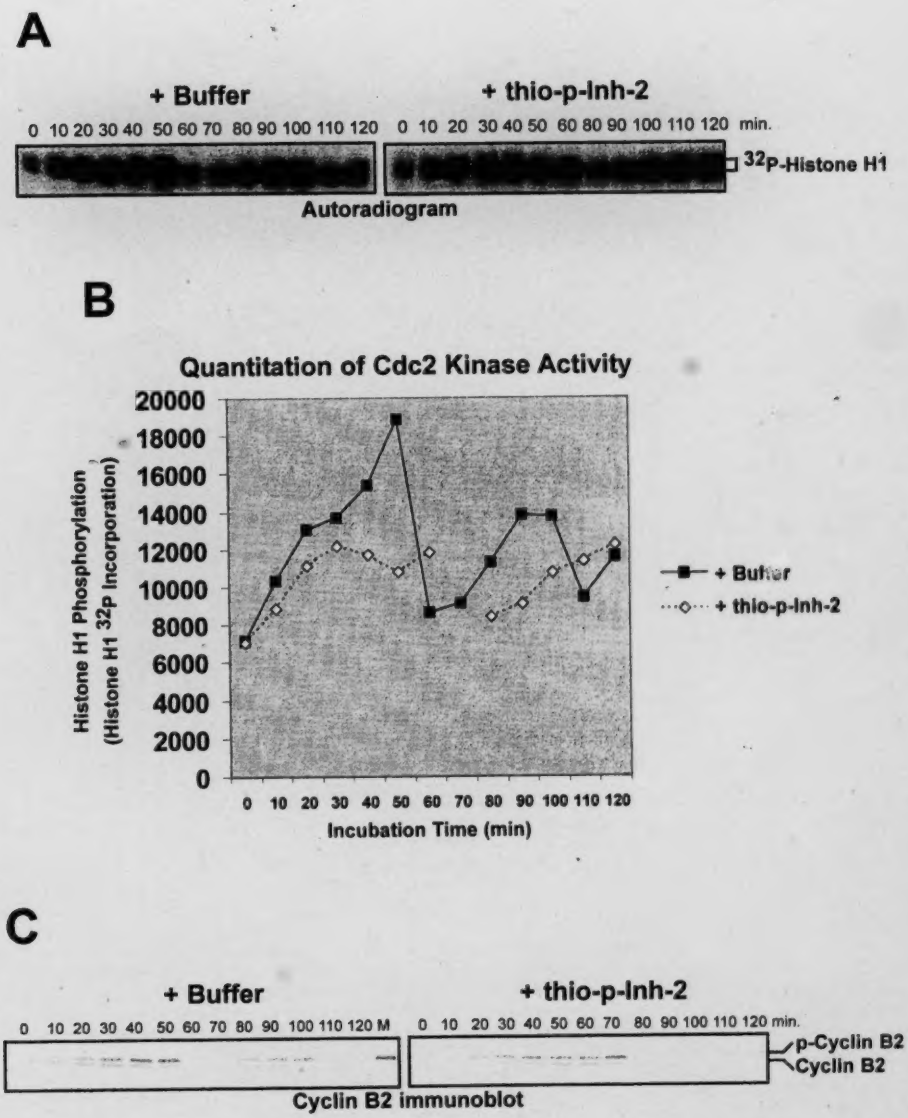
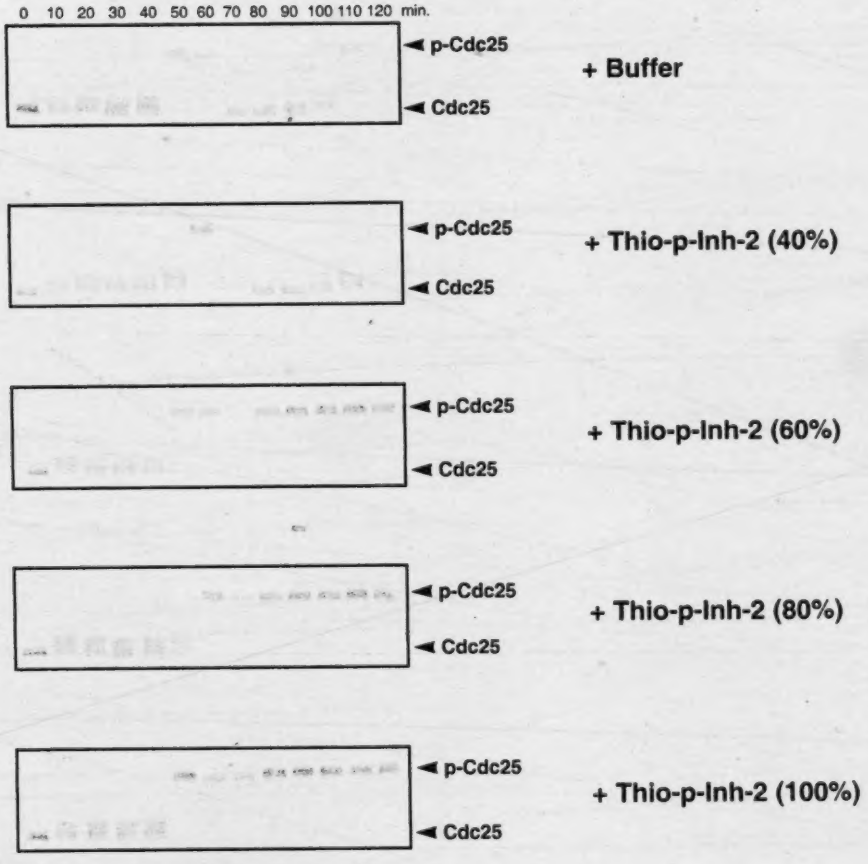


Figure 14: Thiophosphorylated Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Thiophosphorylated Inh-2 in the *Xenopus* Cycling Egg Extract

A *Xenopus* cycling egg extract was prepared, and either buffer or different concentrations of thiophosphorylated rabbit Inh-2 protein was added to 10% (v/v) of the final reaction. Samples were analyzed by SDS-PAGE followed by immunoblotting with Cdc25 antibody. The p-Cdc25 arrow denotes the hyperphosphorylated form of this protein.

Figure 14: Thiophosphorylated Rabbit Inh-2 M-phase Arrest is Dependent on the Concentration of Thiophosphorylated Inh-2 in the *Xenopus* Cycling Egg Extract



Cdc25 immunoblot

Figure 15: The Abilities of Inh-2 and Thiophosphorylated Inh-2 in Inducing M-phase Arrest are Similar

A *Xenopus* cycling egg extract was prepared and either buffer or different concentrations of rabbit thiophosphorylated Inh-2 or rabbit Inh-2 protein was added to 10% (v/v) of the final reaction. The final concentration of proteins in the reactions ranged from 2.6 to 1.6 μM. The reactions were incubated at 21-23°C and samples were taken at 0, 40, 50, 60, 70, and 80 min. Samples were analyzed by SDS-PAGE followed by immunoblotting. The upper panel of each pair shows the Cdc25 immunoblot and the lower panel, the [anti-phosphotyrosine (4G10)] p42MAPK tyrosine phosphorylation immunoblot.

A) The result of the reaction to which buffer was added is shown.
B) The results of the reactions to which different concentrations of Inh-2 were added are shown.
C) The results of the reactions to which different concentrations of thiophosphorylated Inh-2 were added are shown.

Figure 15: The Abilities of Inh-2 and Thiophosphorylated Inh-2 in Inducing M-phase Arrest are Similar

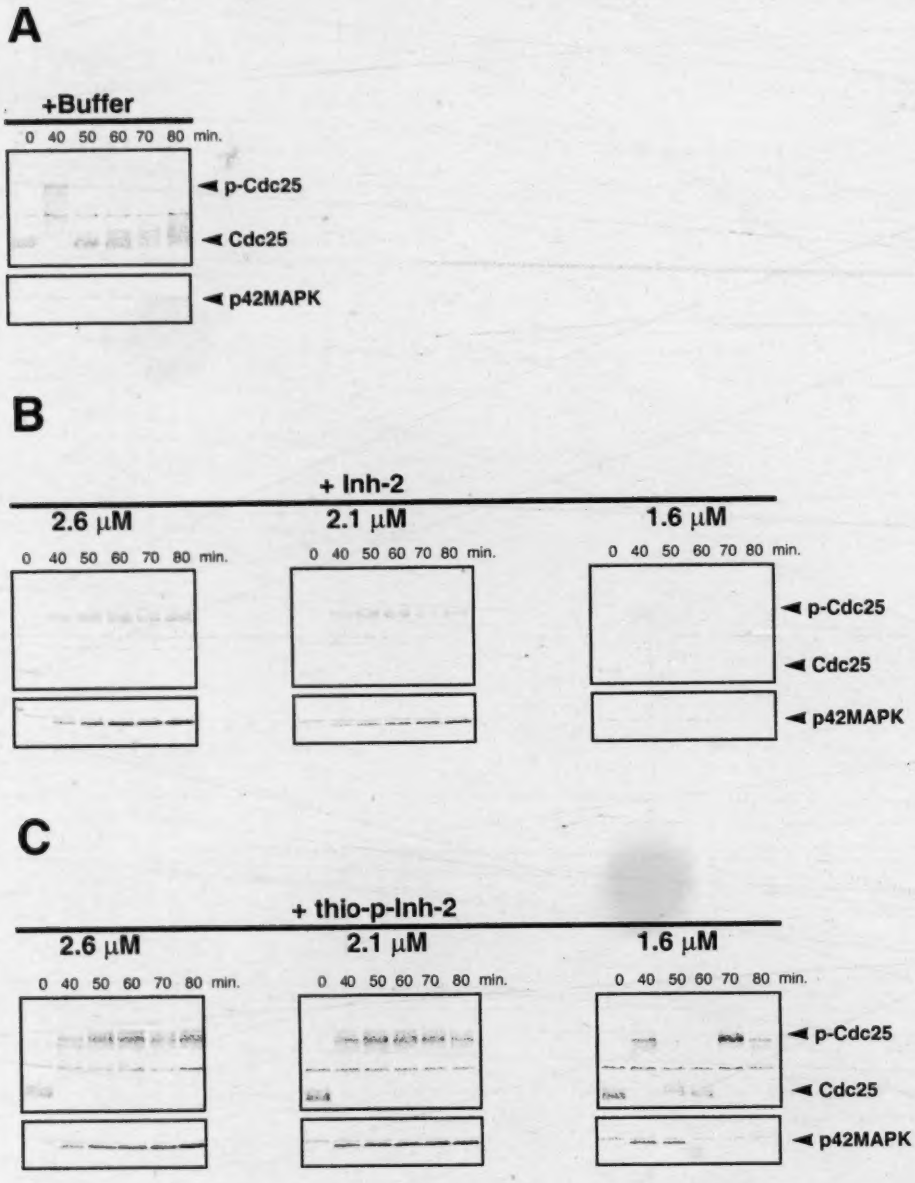


Figure 16: The Rabbit and the Human Inh-2 Amino Acid (a.a.) Sequences Have a High Degree of Identity with the *Xenopus* Inh-2 a.a. Sequence

The rabbit and the human Inh-2 protein amino acid (a.a.) sequences were compared with the partial *Xenopus* Inh-2 a.a. sequence. The partial nucleotide sequence of *Xenopus* Inh-2 (Genbank accession number: BI444584) was translated to yield the predicted *Xenopus* Inh-2 a.a. sequence. The sequence was then compared to the rabbit (Genbank accession number: M95581) and the human (SWISS-PROT accession number: P41236) Inh-2 protein amino acid (a.a.) sequences. The balloon outlines the location of the p90Rsk-1 phosphorylation consensus sites (C.S.) of the rabbit (a.a. 25-28) and the *Xenopus* (a.a. 24-27) Inh-2 proteins which the human (a.a. 25-28) Inh-2 protein does not have. The black shaded area denotes the identical amino acids found in all three sequences.

Figure 16: The Rabbit and the Human Inh-2 Amino Acid (a.a.) Sequences Have a High Degree of Identity with the *Xenopus* Inh-2 a.a. Sequence

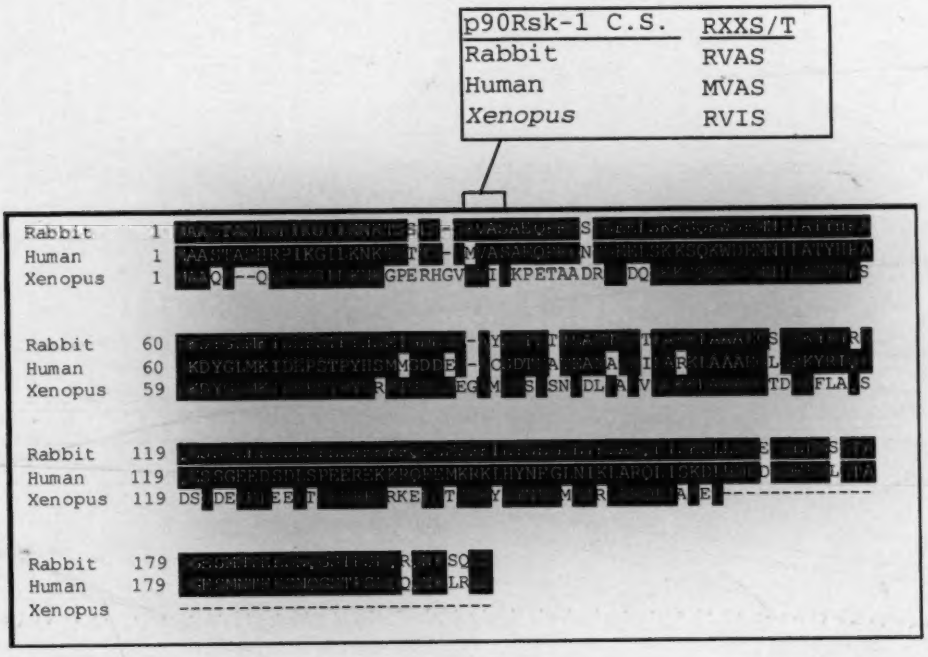


Figure 17: Active pp90Rsk-1 from M-phase Arrested Unfertilized Egg Extracts Does Not Phosphorylate Human Inh-2 Protein *in vitro*

A) The p90Rsk-1 protein was immunoprecipitated (IP) using anti-Rsk-1 antibody from an extract of M-phase-arrested, unfertilized eggs (M). These immunoprecipitates were then used in an *in vitro* kinase assay. The kinase reactions were done in presence of γ-^{35}S-ATP and using both human and rabbit Inh-2 as a substrate. The reactions were incubated for 25 hr at room temperature. At the end of incubation, the reactions were centrifuged and the supernatant (soluble portion) was removed from the pellet (beads). The supernatant samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried.

B) The autoradiogram of the gel in Fig. 17A) is shown.

C) The graph represents the quantitation of ^{35}S incorporation into phosphorylated Inh-2 proteins by phosphorimager.

Figure 17: Active pp90Rsk-1 from M-phase Arrested Unfertilized Egg Extracts Does Not Phosphorylate Human Inh-2 Protein *in vitro*

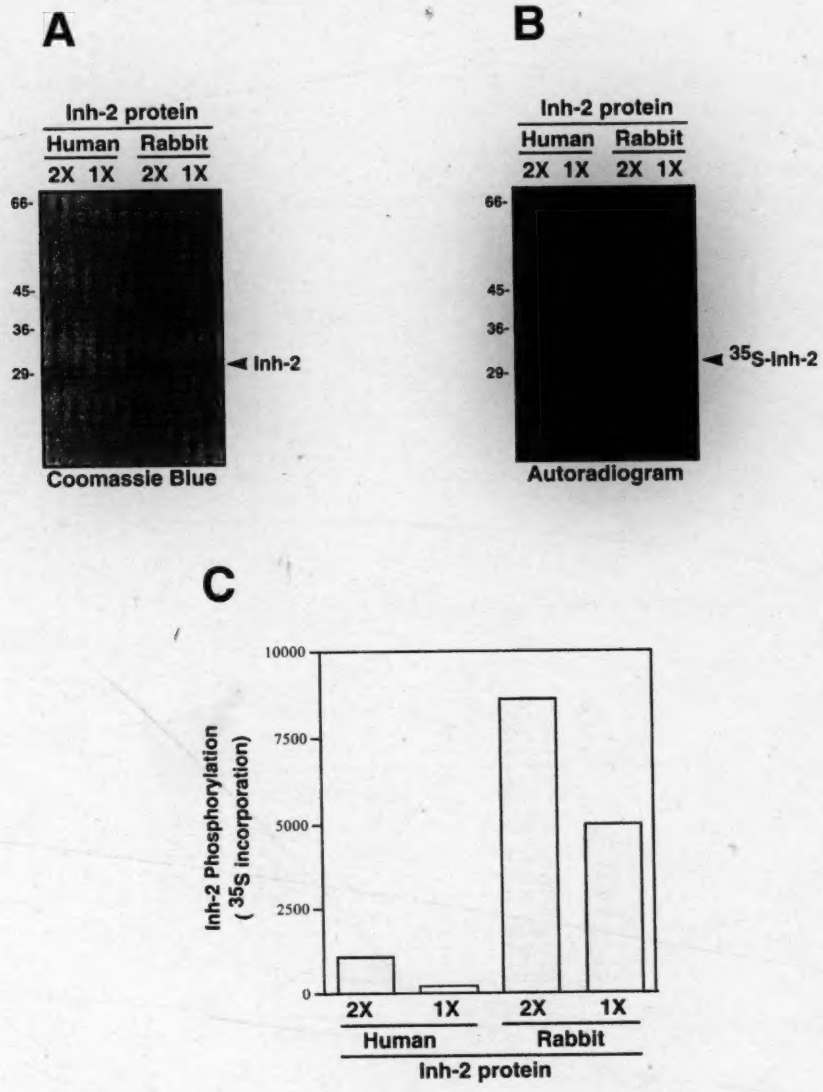


Figure 18: Addition of Human Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extract

A *Xenopus* cycling egg extract was prepared and either buffer or human Inh-2 protein was added to 14% (v/v) of the reaction (Note: the buffer addition results are from the same experiment as in Fig. 5A). Samples were analyzed by SDS-PAGE followed by immunoblotting. The top panel shows a Cdc25 immunoblot, and second panel, p42MAPK tyrosine phosphorylation (4G10) immunoblot. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M). The p-Cdc25 arrow denotes the hyperphosphorylated form of this protein.

Figure 18: Addition of Human Inh-2 Protein Induces an M-phase Arrest in *Xenopus* Cycling Egg Extract

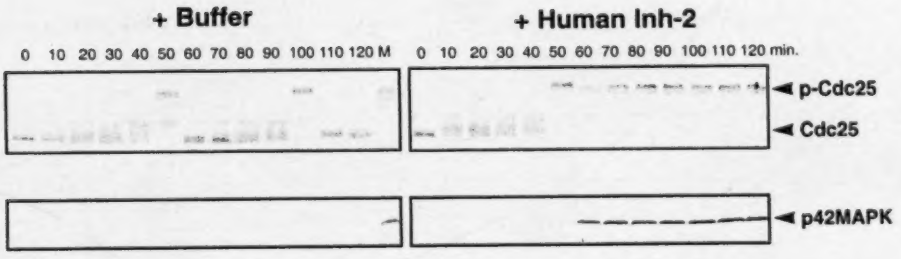


Figure 19: Cdc2 Kinase Activity Levels Fall and Cyclin B is Degraded Followed by an Increase in the Levels of Both in the Human Inh-2 Induced M-phase Arrested Egg Extracts

A) The samples taken from the experiment shown in Fig. 18 were assayed for Cdc2 kinase activity (Note: the buffer addition results are from the same experiment as in Fig. 6A). The histone H1 kinase assay was done in presence of γ-^{32}P-ATP and histone H1 as an exogenous substrate. Kinase reactions were analyzed by SDS-PAGE followed by autoradiography.

B) The graph represents the quantitation of ^{32}P incorporated into phosphorylated histone H1 by phosphorimager.

C) The samples from the experiment shown in Fig. 19A) were immunoblotted with anti-Cyclin B2 antibody. The letter M on the last lane in the buffer addition denotes an extract of M-phase-arrested, unfertilized eggs (M).

Figure 19: Cdc2 Kinase Activity Levels Fall and Cyclin B is Degraded Followed by an Increase in the Levels of Both in the Human Inh-2 Induced M-phase Arrested Egg Extracts

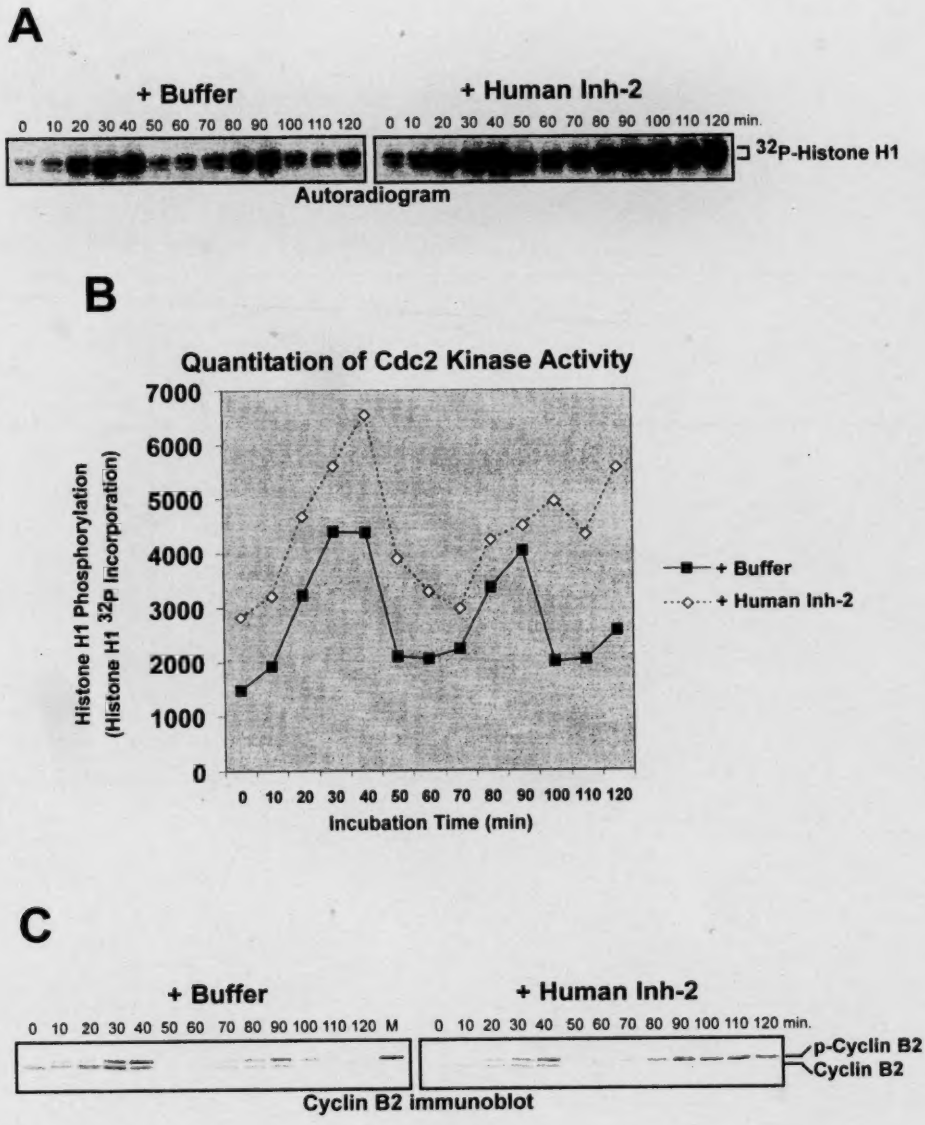

A
+ Buffer
+ Human Inh-2
0 10 20 30 40 50 60 70 80 90 100 110 120
0 10 20 30 40 50 60 70 80 90 100 110 120 min.
32P-Histone H1
Autoradiogram
B
Quantitation of Cdc2 Kinase Activity
Histone H1 Phosphorylation (Histone H1 32P Incorporation)
7000
6000
5000
4000
3000
2000
1000
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Incubation Time (min)
+ Buffer
+ Human Inh-2
C
+ Buffer
+ Human Inh-2
0 10 20 30 40 50 60 70 80 90 100 110 120 M
0 10 20 30 40 50 60 70 80 90 100 110 120 min.
p-Cyclin B2
Cyclin B2
Cyclin B2 immunoblot

Figure 20: Human Inh-2 Antibody Does Not Specifically Recognize Endogenous *Xenopus* Inh-2 Protein on Western Blots

A) A *Xenopus* cycling egg extract was prepared and was added to 2x sample buffer and then the sample was boiled. For the human Inh-2 protein sample, 10 ng of human Inh-2 protein was used. The samples were analyzed by SDS-PAGE and analyzed by immunoblotting with anti-(human) Inh-2 antibody.

B) A *Xenopus* cycling egg extract was prepared and either anti-(human) Inh-2 or anti-Cyclin B1 antibody was used for immunoprecipitation (IP). The anti-Cyclin B1 antibody was used as a negative control. For Inh-2 protein gel lanes, 7.5 ng of rabbit Inh-2 protein and 12 ng of human Inh-2 protein was mixed with 2x sample buffer, the samples were boiled, and then loaded on gels. The samples were analyzed by SDS-PAGE and analyzed by immunoblotting with anti-(human) Inh-2 antibody. Upper arrow indicates human Inh-2 and lower arrow indicates rabbit Inh-2.

Figure 20: Human Inh-2 Antibody Does Not Specifically Recognize Endogenous *Xenopus* Inh-2 Protein on Western Blots

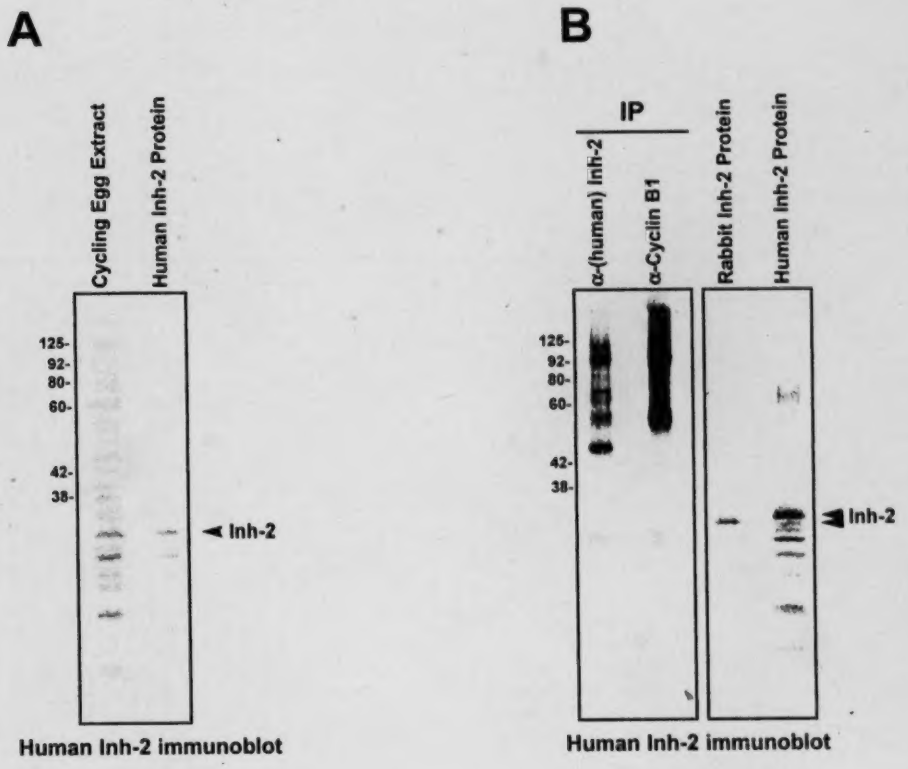


Figure 21: Human Inh-2 Antibody Can Immunoprecipitate Two Phosphorylated Proteins from the *Xenopus* Egg Extract with Molecular Weights Similar to Rabbit Inh-2 Protein

A) The p90Rsk-1 protein was immunoprecipitated (IP) using anti-Rsk-1 antibody from an extract of interphase-arrested, activated eggs (I) or an extract of M-phase-arrested, unfertilized eggs (M). The Cyclin B2/Cdc2 was immunoprecipitated (IP) using anti-Cyclin B2 antibody from an extract of M-phase-arrested, unfertilized eggs (M). These immunoprecipitates were then used in an *in vitro* kinase assay. The kinase reactions (lanes 1-4) were done in presence of γ-^{32}P-ATP and the supernatant of boiled cycling egg extract. The reactions were incubated for 2 hr at 23-24°C. At the end of incubation, the reactions were centrifuged and the supernatant (soluble portion) was removed from the pellet (beads). The supernatant samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried.

B) The autoradiogram of the gel in Fig. 21A) is shown.

C) The anti-human Inh-2 antibody was used to immunoprecipitate (IP) from the kinase reactions from Fig. 21A) (lanes 1-3). For Fig. 21C) lane 4, an *in vitro* kinase reaction was done with p90Rsk-1 IP from an extract of M-phase-arrested, unfertilized eggs (M) in presence of γ-^{32}P-ATP and rabbit Inh-2 as a substrate. The reaction was incubated for 2 hr at 23-24°C. At the end of incubation, the supernatant (soluble portion) was removed from the pellet (beads) and the anti-human Inh-2 antibody was used to immunoprecipitate (IP) from the supernatant. The IP samples were analyzed by SDS-PAGE. The gels were stained with Coomassie blue, destained and then dried. The IgG (HC) arrow denotes the sheep antibody heavy chain IgG and the IgG (LC) arrow denotes the light chain IgG.

D) The autoradiogram of the gel in Fig. 21C) is shown. Upper bracket: lane 4 and lower bracket: lane 2.

Figure 21: Human Inh-2 Antibody Can Immunoprecipitate Two Phosphorylated Proteins from the *Xenopus* Egg Extract with Molecular Weights Similar to Rabbit Inh-2 Protein

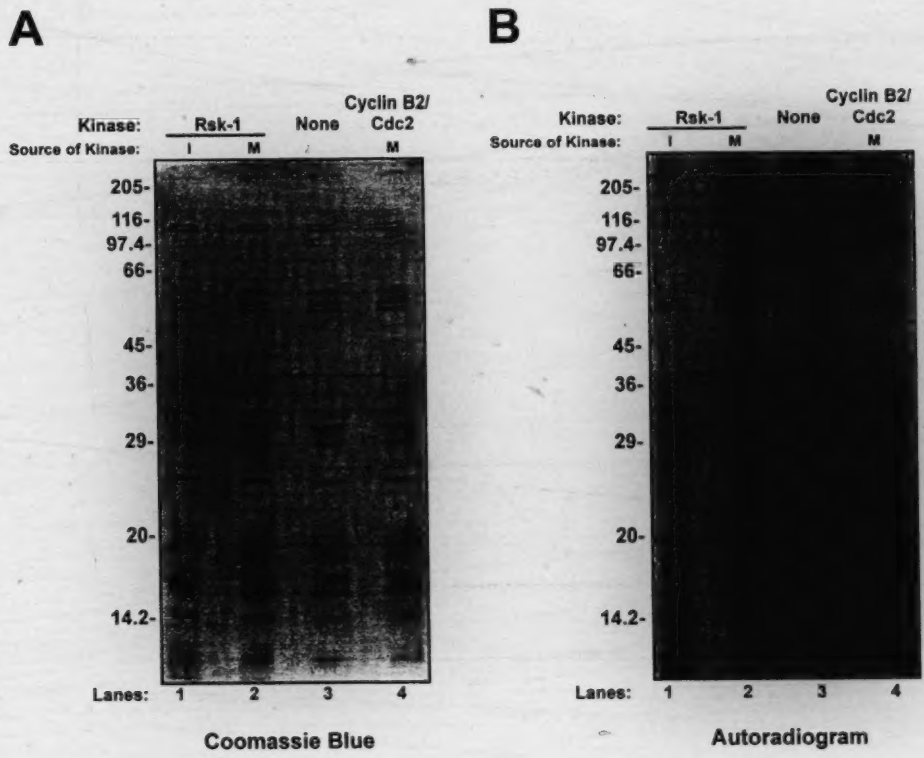


Figure 21:

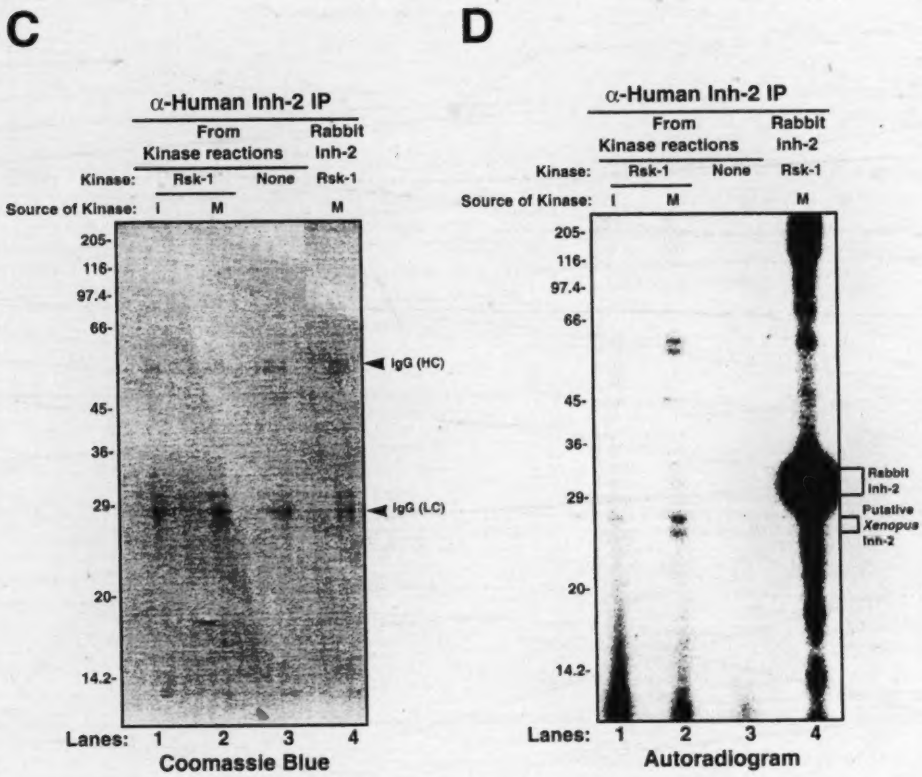

C
α-Human Inh-2 IP
From Kinase reactions
Rabbit Inh-2
Kinase:
Rsk-1
None
Rsk-1
Source of Kinase:
I
M
M
205-
116-
97.4-
66-
45-
36-
29-
20-
14.2-
IgG (HC)
IgG (LC)
Lanes: 1 2 3 4
Coomassie Blue
D
α-Human Inh-2 IP
From Kinase reactions
Rabbit Inh-2
Kinase:
Rsk-1
None
Rsk-1
Source of Kinase:
I
M
M
205-
116-
97.4-
66-
45-
36-
29-
20-
14.2-
Rabbit Inh-2
Putative Xenopus Inh-2
Lanes: 1 2 3 4
Autoradiogram

Figure 22: Cyclin B2/Cdc2 Kinase Immunoprecipitated from Extracts of M-phase Arrested Unfertilized Eggs Phosphorylates Histone H1, PP1 (WT) and PP1 (R308A) but not PP1 (T311D) *in vitro*

A) The Cyclin B2 protein was immunoprecipitated (IP) using anti-Cyclin B2 antibody from two different extracts of M-phase-arrested, unfertilized eggs (Batches 1, 2). These immunoprecipitates were then used in an *in vitro* kinase assay. The samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried. The autoradiogram of the gel is shown. Batches refer to two separate extracts made from different batches of eggs.

B) 40The graph represents the quantitation of ^{32}P incorporated into phosphorylated histone H1 by phosphorimager.

C) Cyclin B2/Cdc2 IPs (obtained as described in Fig. 22A) were then used in an *in vitro* kinase assay. The kinase reactions were done in presence of γ-^{32}P-ATP and substrates human γ-PP1 (WT), mutated PP1 at amino acid 308 where arginine was changed to alanine (R308A), and amino acid 311, where threonine was changed to aspartic acid (T311D). After incubation, the samples were centrifuged and the supernatant (soluble portion) was removed from the pellet (beads). The pellets samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue, destained and then dried. The IgG (HC) arrow denotes the sheep antibody heavy chain IgG and the IgG (LC) arrow denotes the light chain IgG.

D) The autoradiogram of the gel in Fig. 22C) is shown.

E) The graph represents the quantitation of ^{32}P incorporated into phosphorylated PP1 by phosphorimager.

Figure 22: Cyclin B2/Cdc2 Kinase Immunoprecipitated from Extracts of M-phase Arrested Unfertilized Eggs Phosphorylates Histone H1, PP1(WT) and PP1 (R308A) but not PP1 (T311D) *in vitro*

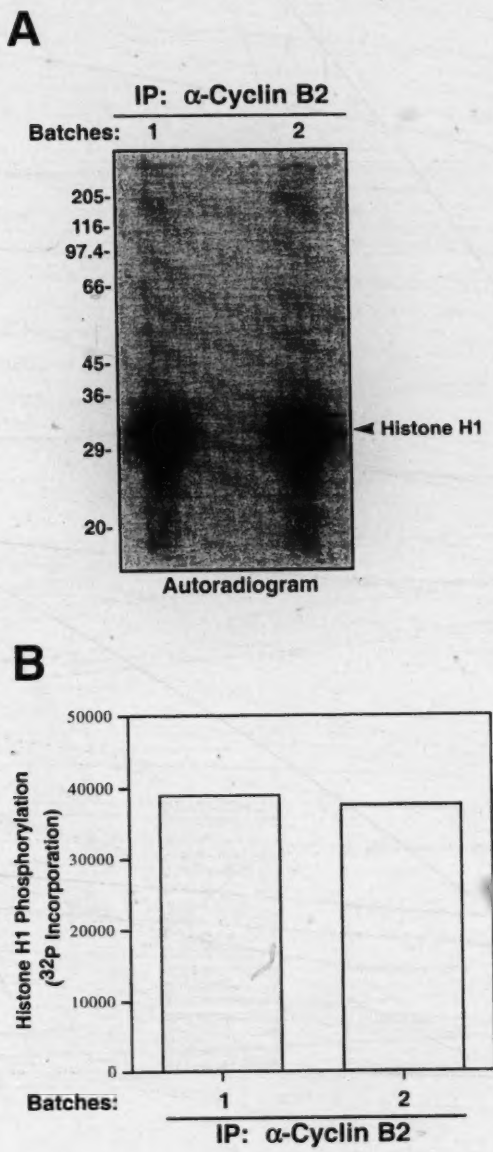


Figure 22:

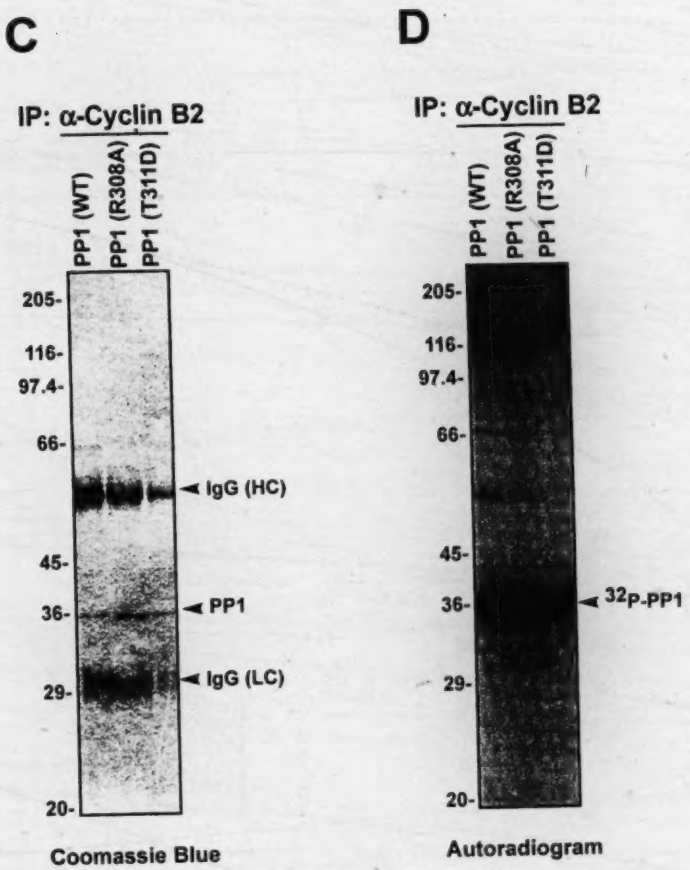
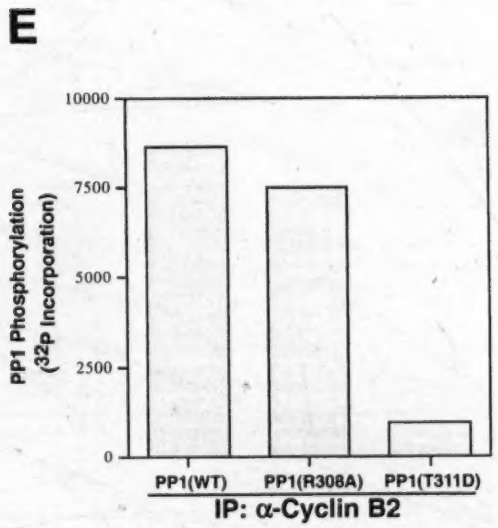

C
IP: α-Cyclin B2
PP1 (WT)
PP1 (R308A)
PP1 (T311D)
205-
116-
97.4-
66-
45-
36-
29-
20-
IgG (HC)
PP1
IgG (LC)
Coomassie Blue
D
IP: α-Cyclin B2
PP1 (WT)
PP1 (R308A)
PP1 (T311D)
205-
116-
97.4-
66-
45-
36-
29-
20-
32P-PP1
Autoradiogram

Figure 22:


E
10000
7500
5000
2500
0
PP1 Phosphorylation
(^{32}P Incorporation)
PP1(WT)
PP1(R308A)
PP1(T311D)
IP: α-Cyclin B2

CHAPTER 6:

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